THE ACTIVATION OF KININS BY ANTIGEN-ANTIBODY REACTIONS

Thesis
Presented for the Degree of
Doctor of Philosophy
in the University of Edinburgh

by

SITES CHANDRA LAHIRI

Department of Pharmacology
University of Edinburgh Medical School

October 1962

STATEMENT IN TERMS OF Ph.D. REGULATION 13
OF THE UNIVERSITY OF EDINBURGH

Some of the results described in this thesis were presented as a preliminary communication before the Physiological Society in November 1961. (Brocklehurst, W.E. and Lahiri, S.C. (1962): J. Physiol., London, 160, 15P)

CONTENTS

PART II: EXPERIMENTAL

LIST OF ILLUSTRATIONS

PART I

SYNOPSIS

SYNOPSIS

The present work was undertaken to find out whether bradykinin played any part in anaphylaxis. The association of anaphylaxis with increased proteolytic activity pointed in this direction; further, the pharmacodynamic effects of bradykinin correspond to certain features of anaphylaxis. Only the antigen-antibody reaction of immediate-type anaphylaxis was studied.

Certain special problems are inherent in such a study. Spontaneous formation and destruction of bradykinin in blood are known to occur very readily. Further, certain experimental manipulations such as the use of a thermo-cautery on skin would activate proteolytic enzymes with resultant formation and/or destruction of bradykinin. Particular care was therefore necessary to ensure that experimental manipulations by themselves did not influence the estimation of bradykinin levels.

A method which neither forms nor destroys bradykinin was developed for estimation of blood bradykinin content. Using this, it was found that following challenge, there is a rapid but short-lived rise in blood bradykinin level in the sensitized guinea-pig, rat and rabbit. The increased activity in the post-shock samples was identified as bradykinin-like by parallel quantitative assay, relaxation of the rat duodenum, and destruction by chymotrypsin. In the rat it was shown that even when the actions of histamine

and 5-hydroxytryptamine are excluded by means of antagonists, anaphylactic shock is followed by a fall in blood pressure, similar to that produced by bradykinin. The participation of bradykinin in anaphylaxis was confirmed by the observation that considerable depletion of plasma bradykininogen content occurs in prolonged anaphylactic shock. In order to demonstrate this, a technique was developed which gave a high yield of bradykininogen from small samples of blood. The amount of bradykininogen used up represents much more potential bradykinin than the peak bradykinin level would indicate. This points to a large turnover of bradykinin in such circumstances due to widely distributed kininase (i.e. bradykinin-destroying) activity.

The release of bradykinin in vitro was also studied by challenging fresh blood of guinea-pig and rat, and blood-free isolated preparations of guinea-pig lung and skin. A skin perfusion method was specially devised to avoid activation of proteolytic enzymes. When shed blood is challenged in vitro, no rise in blood bradykinin occurs. When isolated preparations of blood-free lung and skin are employed, bradykinin itself is not released, but the bradykinin-forming activity (BKF) of the perfusate is rapidly and markedly increased. This was estimated by measuring the amount of bradykinin formed under standard conditions by incubation of perfusate with the bradykininogen present in heat-treated dog plasma pseudoglobulin. BKF release is initiated by antigen-antibody reaction itself, and not the attendant histamine release, as shown by the

failure of administered histamine to induce BKF release.

BKF is comparatively resistant to heat but it is destroyed by boiling. Its optimum pH is 6.5 - 7.0. It is not inhibited by the presence of disodium ethylenediamine tetra-acetic acid (EDTA) or hexadimethrine bromide. Thus BKF of lung tissue differs from the bradykinin-forming enzyme of human plasma. Soya bean trypsin inhibitor (SBTI) suppresses BKF activity.

The kininase activity of normal lung perfusate is thermolabile and is not markedly inhibited with EDTA, hexadimethrine bromide or SBTI. The optimum pH value is 7.0 - 7.4, with a sharp drop on the acid side. Thus at pH value 6.5 - 6.8 conditions are most favourable for bradykinin accumulation since formation is maximal and destruction submaximal. This may be important in pathological conditions such as reactive hyperaemia, trauma and burns where bradykinin may well play a role and where local release of acid metabolites would facilitate its occurrence. Bronchospasm and circulatory stasis might produce similar lowering of pH likely to lead to raised levels of bradykinin.

These findings have been incorporated in a hypothetical scheme illustrating the dynamic state of bradykinin in sensitised tissue challenged <u>in vivo</u> by antigen (fig. 30, p.150).

REVIEW

Since the recognition of bradykinin as a separate entity and its recent synthesis, it has attracted wide attention. An increasingly large volume of work has been and is being done to elucidate its occurrence, metabolism and role in health and disease. The present investigations were aimed at exploration of its role, if any, in immediate-type anaphylaxis. In this chapter an attempt has been made to give a pertinent résumé of the pharmacology of bradykinin and a summary of present knowledge concerning biochemical events of anaphylaxis.

BRADYKININ

The appearance in blood of a substance which produces vasodilatation and slow contraction in plain muscle has been known for some time. Werle, Götze and Keppler (1937) reported that kallikrein, acting on blood *in vitro*, formed a smooth muscle stimulating substance. It had been shown earlier that a vasodilator substance 'Frühgift' was formed spontaneously from cat platelet suspension (Freund, 1920, 1921). Intravenous saliva also has been known to lower blood pressure (Feldberg and Guimarais, 1935). It was thought that protein split products produce these effects in the above cases. The distinct identity of bradykinin was established when Rocha e Silva, Beraldo and Rosenfeld (1949) identified a substance formed by incubating snake venom or trypsin with ox serum, as distinct from histamine and acetylcholine. This produced a slow contraction of isolated guinea-pig ileum preparation, and was accordingly

named bradykinin.

Physical and Chemical properties:

Bradykinin is very stable to heat, and resists prolonged boiling with distilled water or dilute acid. It resists 0.1 N NaOH for two hours at room temperature, but on boiling it is destroyed in a few minutes. It is freely dialysable, is soluble in ethanol, but not in anhydrous acetone or ether. It gives a strong ninhydrin reaction. Using the procedure of stepwise degradation and separation of the various products by two-dimensional chromatography, bradykinin has been found to be a nonapeptide with a molecular weight of 1131 and the following amino-acid structure: Arg. Pro. Pro. Gly. Phe. Ser. Pro. Phe. Arg. (Elliott, Lewis and Horton, 1960). Synthesis by Boissonas and his co-workers has confirmed this structure (Boissonas, Guttman and Jaquenoud, 1960a, 1960b; Boissonas, Guttman, Jaquenoud, Konzett and Stürmer, 1960).

PHARMACODYNAMIC PROPERTIES OF BRADYKININ:

The important features are slow contraction of smooth muscle, vasodilatation, increased capillary permeability, production of pain and leucocyte migration.

Bradykinin produces a contraction in many isolated smooth muscle preparations - rat uterus, guinea-pig ileum, rat stomach, rabbit duodenum, rat colon and guinea-pig uterus. Rat uterus is the most sensitive preparation and contracts at 10^{-10} g/ml. (approximately 10^{-10} M). The slow onset of contraction is characteristic of the

substance. In preparations like rabbit intestine with a spontaneous motility of its own, there is a transient phase of relaxation, which is followed by contraction. Rat duodenum, however, has a purely relaxant response, without any contractile effect. This distinguishes bradykinin from other plain muscle stimulating substances, and helps in its identification. It has no milk ejection effect (Berde and Cerletti, 1961).

Bradykinin lowers blood pressure in various species - rabbit (0.05 µg/kg), dog, rat and guinea-pig (0.2 µg/kg), cat (0.5 µg/kg) and rooster (40-80 µg/kg) (Konzett and Stürmer, 1960). This drop in blood pressure is due to capillary dilatation. The hypotensive response in cat is accompanied by an increase in kidney volume, as shown by renal plethysmography.

The vasodilator activity of bradykinin has been observed in animals and man (Alfonso, Rowe, Castillo, Lowe and Crumpton, 1962). In man, the vasodilator effect is greater after interarterial administration than when given i.v.; this has been attributed to varying rates of destruction (Kidd and Lewis, 1960; Fox, Goldsmith, Kidd and Lewis, 1961). On a molar basis, bradykinin is a more potent vasodilator than acetylcholine, histamine or isoprenaline.

Rocha e Silva, Corrado and Ramos (1960) found that hexamethonium did not influence significantly the hypotension produced by bradykinin, though chlorpromazine, dibenzyline, apresoline and reserpine prolonged the duration

of this hypotension, cocaine reduced the extent and duration of hypotension, but when followed by dibenzyline, this effect was not manifested. All these effects would seem to be due to modification of the normal reflex vasoconstriction or circulatory noradrenaline.

Bradykinin enhances capillary permeability. This can be seen in the guinea-pig blueing test (Bhoola, Calle and Schachter, 1960) and in the rat paw oedema test (Sturmer and Cerletti, 1961).

In keeping with its plain muscle stimulant activity, the general effect of bradykinin on the respiratory system is that of bronchoconstriction, as has been shown in vivo in guinea-pig lung and in spinal cat (Collier, Holgate, Schachter and Shorley, 1960; Konzett and Stürmer, 1960). Isolated bronchioles are surprisingly unreactive. Collier et al (1960) have mentioned a bronchodilator component of bradykinin activity in some species which can be unmasked when the bronchoconstrictor component is antagonized by antipyretic antagonists. In dog, the respiratory rate and volume rises (Konzett and Stürmer, 1960); in the bronchi, either some dilatation occurs, or there is no effect (Waaler, 1961). Herxheimer & Streseman (1961) have reported that bradykinin aerosol produces moderate bronchoconstriction in asthmatic man, while there is no effect in normal man. Pure bradykinin, applied to blister base, produces pain (Elliott, Horton and Lewis, 1960). Pericapillary non-myelinated fibres and branching varicose terminals around blood vessels may be similar to free branching 'pain' receptors

in cutaneous epithelium (Weddell and Pallie, 1954; Lim, 1960). Braun, Guzman, Horton, Lim and Potter (1960) found that intra-arterial injection of bradykinin to lightly anaesthetised dogs produces vocalisation, hyperpnoea and vasomotor changes which they have attrtibuted to the pain-producing effect. Guzman, Braun and Lim (1962) suggest that the response is a reflex phenomenon, arising from the pain-endings.

The central effects of bradykinin do not seem to have been investigated in much detail. Rocha e Silva (1960) refers to some unpublished experiments of Corrado and Ramos where bradykinin given via intraventricular cannula to unanaesthetised cat caused hypotension, sometimes tranquillisation and relaxation of the nictitating membrane, though some animals became restless and went into convulsions. Čapek (1961) reported that under similar circumstances motor disturbances - muscular twitches leading to tonic convulsions - occurred. Hyperventilation, salivation, micturition and mydriasis occurred. Behaviour changes also appeared - the slightest touch caused continuous phonation and the animals lay down in bizarre positions. Similar events occurred in mice also on intracerebral injection of bradykinin. Bradykinin increased the convulsive activity of leptazol and strychnine.

Bradykinin vis-à-vis Kallidin:

Kallidin, produced by incubating kallikrein with bovine and dog serum was found indistinguishable from bradykinin pharmacologically (van Arman and Miller, 1961).

Pierce and Webster (1961) found that kallidin consists of kallidin I, which has the same chemical structure as bradykinin, and kallidin II, which is a decapeptide with a closely similar amino-acid sequence - H. Lys. Arg. Pro. Pro. Gly. Phe. Ser. Pro. Phe. Arg. Kallidin II has recently been synthesized (Nicolaides and De Wald, 1962). Kallidin II has the same pharmacodynamic properties as bradykinin, though it is only half as active (Pierce and Webster, 1961).

ANTAGONIST DRUGS:

Acetylsalicylic acid, phenylbutazone and amidopyrine have been found to antagonize bradykinin bronchoconstriction in vivo in guinea-pig, in doses in which bronchoconstriction due to histamine and 5-hydroxytryptamine were not affected (Collier, Holgate, Schachter and Shorley, 1960; Collier and Shorley, 1960). Increasing doses of bradykinin overcame this antagonism which could be restored with increasing doses of the antagonist. When other test objects were employed, no antagonism could be shown with these compounds except as part of a general depressant response. To explain this, the above workers have suggested that bradykinin may act on more than one kind of receptor, whereas antagonists have a narrower range of action. There does not seem to be enough evidence for any satisfactory conclusion, as antagonism could be shown only on living in vivo plain muscle which is more sensitive in vivo than in vitro to bradykinin whereas most other plain muscle

preparations (intestine, uterus) are more sensitive in vitro. Further information is clearly desirable in this field.

Phenylbutazone has been reported to suppress the lowering of blood pressure in rabbit by bradykinin, but the high dosage employed (100 mg/kg) detracts from the significance of these findings (Lecomte, 1959; Lecomte and Troquet, 1960).

Braun, Guzman, Horton, Lim and Potter (1960) have reported that the algesic response of lightly anaesthetised dogs to bradykinin (vocalisation, hyperpnoea and cardio-vascular changes) are partially or completely blocked by acetylsalicylic acid or N-acetyl-para-aminophenol.

SOURCE OF BRADYKININ:

Plasma contains the precursor of bradykinin in the euglobulin fraction, but it has never been isolated and characterised. Bradykinin precursor is usually called bradykininogen, and more than one protein may be involved. In the absence of adequate fractionation methods, bradykinin-forming enzymes have so far been tested only on relatively crude samples of bradykininogen. Quantitative studies in bradykinin formation are difficult in these samples because several reactions proceed simultaneously: (a) formation of bradykinin, (b) destruction of bradykinin due to kininase activity, and (c) inhibition of bradykinin-forming enzyme by an inhibitor, also present in the substrate.

Attempts have been made to dissociate bradykininogen

from these interfering substances. One approach has been to heat the material, with or without acid-denaturation. The enzyme inhibitor present in plasma is said to be destroyed by heating at 56° for 3 hours (Werle, 1934; Werle, Götze and Keppler, 1937). Lewis (1960) states that this method reduces but does not eliminate the inactivator. Van Arman (1955) denatured plasma by boiling with acetic acid to eliminate interfering substances. This preparation, however, is unsuitable for comparatively slow-acting enzymes (Lewis, 1960). Horton (1959) found that if dog plasma pseudoglobulin is incubated at 37°C and pH 2 for 15 minutes and then neutralised, kininase and enzyme inhibitor are destroyed. Acidification might be expected to activate kallikreinogen (Frey, Kraut and Werle, 1950) and thus form kinin during such incubation, but Horton showed that the incubation mixture contains the same amount of kinin before and after incubation. Of the substrates available, this preparation is the best at present used for investigation of bradykinin-forming enzymes.

Another approach has been to fractionate plasma to dissociate interfering enzymes from bradykininogen (Rocha e Silva, Beraldo and Rosenfeld, 1949). They precipitated plasma with 50% ammonium sulphate saturation at first but later it was found that better yields were obtained with fractional precipitation between 33% and 46% saturation. However, interfering enzymes were present in this preparation also. In any case, the protein content of pseudoglobulin preparation precipitated between 33% and 46%

ammonium sulphate saturation is quite mixed, the yield from 100 ml serum containing 0.15 g albumin, 0.12 g α_1 globulin, 0.55 g α_2 globulin, and 0.67 g of β- and gamma-globulins combined (Lewis, 1960). The presence of the precursor of a kinin-forming enzyme in serum, which may be kallikreinogen or plasminogen or both, further complicates matters. Plasminogen activators, when added to pseudoglobulin enhance kinin formation.

The globulin fraction containing bradykininogen varies from species to species. Werle (1955) had investigated ox plasma by paper electrophoresis and aqueous elution. He found that bradykininogen occurred in α_2-globulin, but not α_1-globulin or albumin. Kininase activity is associated with this fraction, which reduces its suitability for use as a substrate. Van Arman's experiments (1955) suggest that the β-globulin fraction is more important than α-globulin for bradykinin formation in human serum with Bothrops jararaca venom. In terms of Cohn's fractions, van Arman (1955) found that fraction IV was the only one which formed bradykinin; of this fraction, IV-4 yielded more bradykinin than IV-1. The former contains more β- than α-globulin, while the reverse occurs in the latter. Further support was available from electrophoresis experiments where he found that of the three peaks, α-globulin, β-globulin and albumin, only β-globulin was reduced by bradykinin formation. Preparation of a purified substrate free from enzymes or their precursors is essential before the finer details of bradykinin formation can be elucidated.

It may be possible to isolate bradykininogen with the more recent methods of protein fractionation.

FORMATION OF BRADYKININ:

The physiological and pathological modes of bradykinin formation are not fully known. Trypsin and certain snake venoms were originally employed in the preparation of bradykinin. Apart from these, bradykinin is also formed by kallikrein, plasmin, glass-activation, and dilution of plasma.

Kallikrein:

The presence of a hypotensive substance in urine was first reported by Abelous and Bardier (1909) and Pribram and Herrnheiser (1920). Frey and Kraut (1925) called it 'Kallikrein' after the Greek word καλλίκρεας meaning pancreas, because they thought it was a pancreatic hormone with cardiovascular activity, but later it was recognised to be an enzyme, which forms kallidin from serum globulin. At present kallikrein is not a very specific term and may even cover more than one enzyme.

Kallikrein was formerly assayed by its vasodepressor effect on dog blood pressure. It was found later that the amount of kallidin formed from heated acid-treated dog plasma pseudoglobulin on incubation under standard conditions is proportional to the amount of kallikrein incubated (Horton, 1959). This method is now in general use.

Formerly it was thought that kallikrein was found solely in the pancreas. It is now known that pancreas is neither the sole nor the major source of kallikrein (Beraldo, 1952; Beraldo, Feldberg and Hilton, 1956). Kallikrein is found in considerable amounts in pancreas & salivary gland; a plasma kinin forming enzyme is also found in skin (Fox and Hilton, 1958), cerebrospinal fluid (Chapman and Wolff, 1958) and lachrymal secretion (Lewis, 1959).

Kallikrein produces its pharmacological effects through formation of kallidin which has the same properties as bradykinin. It has been claimed, though not confirmed, that kallikrein by itself, and not via kallidin formation contracts dog intestine.

Blood contains kallikreinogen, an inactive precursor of kallikrein. Frey, Kraut and Werle (1950) suggested that kallikrein from pancreas or salivary glands travelled to the blood stream, either by direct internal secretion, or indirectly by resorption from the intestinal tract. As for the salivary gland, it has no mechanism for internal secretion, nor would kallikrein survive gastric acidity. Werle (1960) found that in a ligated submandibular duct, kallikrein disappears long before histological changes occur. Transfer of salivary gland kallikrein to blood thus seems unlikely. As regards pancreas, there is some evidence against resorption of kallikrein through the intestinal wall (van Winkle, 1941). Endocrine adsorption of pancreatic kallikrein is also unlikely because it has been shown that kallikrein level does not

change with experimentally induced degeneration of β - or other cells.

Different forms of kallikrein:

Kallikrein from different sources have been attributed slightly different properties. "Ornitho-kallikrein" (of avian origin) forms kallidin from avian but not mammalian sera, and mammalian kallikrein acts on mammalian but not avian sera.

Kallikreinogen:

Kallikrein is not present in an active form in blood. It is present in combination with an inactivator. The term 'kallikreinogen' has been applied to this inactive condition. Apart from pancreas, Werle and Vogel (1960a) found kallikreinogen in the wall of the small and large intestine of man, rat, dog, cat and cattle. Pancreas is not a major source of kallikreinogen, since pancreatectomy in dog or rat does not reduce kallikreinogen content of serum or intestine (Werle and Vogel, 1960). The source could be liver, since experimental liver damage with carbon tetrachloride in rat, and liver disease in man, is associated with a low blood kallikreinogen content. The kallikreinogen content of liver is higher than that of blood, so this subject needs further study.

It is not yet known how physiological activation of kallikreinogen takes place. Blood contains very little active kallikrein, but normal urine contains a fair amount of it. Activation of kallikreinogen may be one of the

physiological events in the kidney. Investigating this possibility, Werle and Vogel (1958, 1960b) found that when experimental renal damage is induced in rats with uranyl acetate or mersalyl, kallikrein disappears from urine, but kallikreinogen starts to appear. If the renal damage is reversible, these changes disappear later. Kallikreinogen has also been found in human nephrotic urine (Werle and Vogel, 1958). These authors (1960) have suggested that kallikreinogen is activated by tubules. This appears to be possible, but there are other possibilities - e.g. alteration of pH, tonicity, etc. It would be interesting to know if the glomeruli will pass kallikrein, but not kallikreinogen.

Blood kallikreinogen has been activated in vitro by acidification, acetone treatment, and treatment with papain (Frey, Kraut and Werle, 1950). Acidification to pH 2 releases considerable quantities of kallikrein in blood. As acid metabolites may lower pH locally, this may be of importance in physiological activation, though pH 2 is unlikely to occur in the course of physiological events. Activation of kallikreinogen can also be produced on treatment with acetone or papain.

Kallikrein inactivation:

The existence of a kallikrein inhibitor in blood was realised somewhat fortuitously when Frey and his colleagues found that samples of urine collected after a long period of experimentally induced reflex anuria, and strongly

positive when tested for the presence of blood, had little effect when tested for kallikrein on the cardiovascular system (Frey, Kraut and Werle, 1950). These workers found an inactivator substance in blood and several other tissues. It should be remembered that antiproteolytic activity in blood has been known for many years, and probably is of little specificity.

Kallikrein inactivator effect has been found in other sources also: bovine parotid gland (Kraut and Korbel-Enkhardt, 1957); blood of the vineyard snail Helix pomatia (Werle, Appel and Happ, 1958); and potatoes (Werle and Maier, 1955; Werle, Maier and Löffler, 1951).

Northover and Subramanian (1961) studied the effect of analgesic-antipyretic drugs in vitro on kallidin formation by guinea-pig serum kallikrein or human salivary kallikrein. They found that some of these agents (phenylbutazone, 2:6-dihydroxybenzoic acid, Na α-4-sec-butyl-phenoxypropionate, aspirin and salicylate) inhibited kallidin formation in concentrations which did not affect the smooth muscle stimulant activity of kallidin or bradykinin. These concentrations were much lower than those necessary for general anti-enzyme activity (Lutwak-Mann, 1942).

Plasmin:

Plasmin is a proteolytic enzyme normally present in blood in an inactive form, plasminogen. When this is activated, proteolytic activity of blood increases. In view of the raised proteolytic activity, plasmin was investigated for bradykinin-forming activity by different

workers. Beraldo (1950) showed that plasmin, on incubation with plasma, forms a bradykinin-like substance. Lewis and Work (1957) reported the slow development of bradykinin-like activity on incubation of human plasmin with dog plasma pseudoglobulin, whereas plasminogen, the active precursor had neither proteolytic nor bradykinin-forming properties, though these properties appeared on addition of the specific activator streptokinase. Weight for weight crystalline trypsin has a greater bradykinin-forming activity than plasmin. When amounts of trypsin and plasmin which have equal proteolytic (presumably caseinolytic or fibrinolytic) activity are compared for bradykinin-forming activity, plasmin forms more bradykinin than trypsin (Lewis, 1960). Intravenous plasmin reduces blood pressure in dog, probably due to bradykinin formation. If administered repeatedly, its response is gradually reduced probably due to gradual substrate (i.e. bradykininogen) depletion. After such depletion by plasmin, the hypotensive effect of kallikrein is reduced and vice versa. Eisen (1961) found that when "pre-active" (i.e. plasma not previously subjected to activation processes) human plasma is incubated with plasmin, kinin formation commenced in about thirty seconds, reaching the peak in about five minutes. The activity fell to a low level in 20 to 30 minutes. These workers considered plasmin as a quick-acting enzyme, since it acted as rapidly as glass-activation. Formation of bradykinin by plasmin is inhibited by antiplasmin (Lewis, 1958). Soya bean trypsin inhibitor (Lewis, 1958; Schachter, 1960) inhibits kinin formation by plasmin as also kallikrein; the latter is not

inhibited by ovomucoid trypsin inhibitor (Schachter, 1960).

The possibility of kallikrein being involved in plasmin activity has been explored (Schachter, 1956; Lewis, 1958; Webster and Pierce, 1960). These enzymes can be distinguished by their response to heat and acidity. These are very similar with regard to their relationship to inhibitors, so that they can be distinguished only by the ratio of trypsin inhibitors required to inhibit these enzymes; the approximate ratio of SBTI, OTI and PTI* are 1:10:100,000 for plasma kallikrein as compared to 1.0:0.36: 1.77 for plasmin (Green, 1953). Furthermore, o,o-bis-(2-chloro-ethyl)-o-(2,2-dichlorovinyl) phosphate inhibits plasma kallikrein, but not plasmin.

Werle (1955) found that acetone treatment of plasma activates proteolytic enzymes which act on kallikreinogen, forming kallikrein. Investigating this, Webster and Pierce (1960) suggested that plasmin was not the protease concerned, since ovomucoid did not inhibit acetone activation of plasma kallikrein and acetone did not activate plasminogen. They also observed that SBTI did not inhibit splitting of kallidinogen in the presence of streptokinase, and suggest that it is possible that streptokinase activates not only plasmin but also other kallidinogen-splitting proteases, though any such have not been identified so far.

Activation of plasminogen:

A number of substances activate plasminogen to plasmin. These may be present in bacterial cells (staphylokinase and

* Trypsin inhibitors from soya bean, ovomucoid and pancreas, respectively.

streptokinase), in normal urine (urokinase), in blood and other tissues (Müllertz, 1956). The activators found in skin, lung and brain are insoluble in water and can be brought out in solution only with thiocyanate (Goldhaber, Cornsman and Ormsbee, 1947; Tagnon and Peterman, 1949; Lewis and Ferguson, 1950; Astrup and Sterndorff, 1956; 1956).

When tissue plasmin activator from skin or urokinase is incubated with bradykininogen-containing pseudoglobulin solution, plasma kinin is formed (Lewis, 1959; Horton and Lewis, 1960). However, streptokinase incubated with pseudoglobulin does not form plasma kinin unless plasminogen is present (Lewis, 1959; Horton and Lewis, 1960). This is probably due to the fact that a "pro-activator" is necessary for activating the streptokinase; the "pro-activator" is present in plasminogen, and hence addition of plasminogen sets the activation mechanism in motion (Müllertz and Lassen, 1953; Lewis, 1959; Horton and Lewis, 1960). The proteolytic activity of blood is balanced delicately between plasmin, plasmin inactivator (antiplasmin) and an "anti-inactivator" (Müllertz, 1956). The role of plasmin in the physiological formation of plasma kinin is not yet clear. It has been invoked in glass-activation and dilution-activation, as discussed later. In any event it is activated <u>in vivo</u> in a number of conditions, and could lead to raised levels of bradykinin and thus contribute to the overall picture.

"Quick-acting" and "Slow-acting" bradykinin-forming enzymes:

Lewis mentions (1960) that while the rate of enzymic

conversion depends on substrate concentration - plasma bradykininogen in this case - kallikrein never takes more than 10 to 15 minutes for maximal kinin formation ("quick-acting enzyme") and urokinase, probably acting through plasmin, never less than 20 to 30 minutes ("slow-acting enzyme"). Eisen (1961) does not agree with this division; under his experimental conditions, plasmin could produce maximal bradykinin formation within very few minutes. Hence this division between "quick-acting" and "slow-acting" enzymes will need further exploration and confirmation. The discrepancies are probably due to the very impure enzymes and substrates used.

Glass-activation:

Plasma, blood and inflammatory exudates have been shown to form a pain-producing substance on contact with glass (Armstrong, Dry, Keele and Markham, 1953; Armstrong, Hobbiger, Jepson and Keele, 1953; Armstrong, Jepson, Keele and Stewart, 1954, 1955 and 1957). This substance can be assayed on rat uterus. The activity reaches a peak in a few minutes, but declines by 90% in an hour. After glass-activation and decay, re-activation by a second exposure to glass is not possible, though these samples can activate "pre-active" plasma, thus showing that enzyme or activator was still present after all the substrate was used up. The active substance closely resembles bradykinin. Margolis (1958) postulated that exposure to glass activated an inactive plasma factor

("component A") to an active factor ("contact factor") for which Hageman Factor was essential. Contact factor combines with another factor ("component B") in blood and liberates plasma kinin. Lewis (1958, 1960) suggests that this could be the plasminogen-plasmin system. However, epsilon-aminocaproic acid (ϵAC) does not inhibit glass activation, though it inhibits formation of kinins by plasmin. ϵAC has even some kinin-forming capacity of its own. The speed of glass-activation is also in contrast with the relatively slow action of plasmin.

Activation by dilution:

Schachter (1955) has shown that when serum or plasma of guinea-pig, rabbit, rat, cat, dog and man is diluted optimally with physiological solution, bradykinin-like activity develops and later decays. Plasma previously depleted of bradykininogen by trypsin could not be activated by dilution. Plasminogen is activated by dilution (Macfarlane and Pilling, 1946), and diluted guinea-pig serum has been shown to develop a substance which increases capillary permeability (Mackay, Miles, Schachter and Wilhelm, 1953; Miles and Wilhelm, 1955). Schachter found that dilution-activated plasma and kallikrein are similarly affected by soya bean trypsin inhibitor and by heating at 56°C for 3 hours. He has therefore suggested (1956) that dilution activates kallikreinogen to kallikrein which then proceeds to form plasma kinin.

Recently, Eisen (1961) has reported that both dilution-activation and glass-activation of human plasma are due to the same enzyme. Armstrong et al. (British Pharmacological Society Winter Meeting, 1962) reported that a purified preparation of this enzyme, homogeneous on electrophoresis was antagonised by polybrene, an antagonist of heparin. This distinguishes it from plasmin.

DESTRUCTION OF BRADYKININ BY KININASE:

Kallidin is inactivated on incubation with serum or plasma (Werle, 1955). Kininase activity has been found in the serum of guinea-pig, cat, dog, ox and man, guinea-pig serum being the most potent (Schachter, 1960). Destructive activity was found to be similar in serum and lymph collected from the thoracic duct. In blood, kininase activity has been found in formed elements also (Erdös, Renfrew, Sloane and Wohler, 1962; Schwab, 1962). Werle (1955) found kininase activity in his α_1-globulin prepared by precipitation with ammonium sulphate. Edery and Lewis (1962) found that kininase activity is very much depressed at pH 6.5. Werle (1960) has reported increased serum kininase activity during the terminal weeks of human pregnancy.

Kininase activity has been reported in various organs including kidney and liver. Hamberg and Rocha e Silva (1954) distinguished a bradykininase from a histaminase present in hog kidney. The kininase activity was not associated with oxygen consumption, and the optimum pH was $\geq$ 7.4;

this sets it apart from enzymes like beef spleen cathepsin I which is reported to have pH 5.4 as optimum value (Bergman, 1941). Kininase activity has been found in liver tissue, but the organs in these experiments were not blood-free and it is not known how much was due to blood (Werle, 1955). Chymotrypsin has a potent kininase activity. Early reports attributed kininase activity to trypsin also, but this is now known to be due to chymotrypsin contamination.

Cysteine has been reported to inhibit kininase (Picarelli, Henriques and Oliveira, 1962). As cysteine potentiates bradykinin-induced contraction in plain muscle, any evaluation of its anti-kininase activity needs special care.

ROLE OF BRADYKININ IN NORMAL AND PATHOLOGICAL STATES:

Despite widespread interest and much work, there is as yet no comprehensive picture of the role of bradykinin in normal and pathological states. There is good evidence that it acts in functional vasodilatation of salivary secretion and in sweating, but only suggestive evidence of involvement in various other phenomena.

In functional vasodilatation of salivary secretion:

Milton and Lewis (1956 et seq.) investigated the possibility of bradykinin acting as a mediator for functional vasodilatation accompanying activity in salivary gland and muscle. Employing the technique of intermittent Tyrode perfusion of cat submandibular gland, they found

that when the gland was stimulated by cholinergic or adrenergic drugs or parasympathetic or sympathetic stimulation, the perfusate showed hypotensive activity. Smooth muscle stimulant activity was not present as such in the perfusate, but developed when perfusate was incubated with plasma. The similarity in the time-course of development of hypotensive response and development of smooth muscle stimulant activity suggested the same active principle was responsible for both the properties. When saliva + pseudoglobulin and perfusate + pseudoglobulin mixtures were compared with bradykinin, parallel quantitative tests showed good correspondence and no distinction could be found with simple physicochemical tests. The forming enzyme was evidently present in saliva also, and this gave new significance to older findings (Secker, 1934; Feldberg and Guimarais, 1935) that saliva had hypotensive, but not smooth muscle contractile properties. The small amount of bradykinin-forming activity in perfusate from non-stimulated gland increased 2.5 to 8.5 times after stimulation. Employing the technique of circulatory arrest, Hilton and Lewis (1956) concluded that the enzyme-substrate reaction takes place not in the circulating blood but in the interstitial fluid. Bradykinin precursor has not yet been shown specifically in interstitial fluid, but mammalian lymph, a closely allied material has been shown to contain all the fractions in mammalian plasma (Yoffey and Courtice, 1956). From their experiments, Hilton and Lewis (1956) concluded that when

salivary gland secretes, no matter how it is stimulated to do so, a plasma-kinin forming enzyme escapes into the interstitial spaces of the gland and forms plasma kinin which acts as the mediator for the vasodilatation which accompanies glandular activity.

Vasodilatation in the tongue, produced by stimulation of hypoglossal and chordo-lingual nerves was also investigated (Hilton and Lewis, 1958). Chordo-lingual stimulation was found to increase the enzymic activity twofold or more. The authors considered and excluded antidromic stimulation as the causal factor. They concluded that a bradykinin-forming enzyme, released during stimulation of the tongue, formed bradykinin. Their findings suggested that activation of the glandular, and not muscular, element of the tongue is involved in the process.

Bradykinin and cutaneous blood flow:

Bradykinin has been investigated as a possible mediator of functional vasodilatation of sweating (Fox and Hilton, 1958). Bradykinin-forming activity was found like saliva in human eccrine sweat, but not in the perfusate obtained from the subcutaneous space in man. Subcutaneous perfusate, however, contained bradykinin-like activity. These workers excluded the possibility of bradykinin formation by dilution of plasma with saline by showing that the tyrosine content of the perfusate did not rise. Fox and Hilton (1958) concluded that in human eccrine sweat glands, bradykinin formation produces a

periglandular vasodilatation, and thus plays an important role in sweat secretion; and that forearm vasodilatation during body heating is mainly induced by bradykinin formed by sweat gland activity.

Lewis (1960) has questioned these conclusions. Increased amounts of blood flowing through the capillary bed, however mediated, with diffusion of saline into capillaries, will lead to formation of bradykinin due to dilution-activation. In such a case, the subcutaneous perfusate should contain kinin, but no kinin-forming enzyme. However, if activated sweat glands released enzyme in the periglandular space, the perfusate should contain some kinin-forming enzyme. As the matter stands at present, Fox and Hilton's (1958) experiments have established a role for bradykinin in functional vasodilatation in sweating, but it is not known whether other mechanisms are involved.

Bradykinin-forming activity occurs in other glands e.g. lachrymal and pancreatic. Lewis (1959, 1960) pointed out the wide concurrence of gland activity with bradykinin formation, and suggested that in general functional vasodilatation accompanying glandular activity may be mediated by local formation and release of plasma kinins. Bhoola, May May Yi, Morley and Schachter (1961) do not agree with this suggestion. They have pointed out that guinea-pig saliva does not form kinin from its own plasma, though kinin is formed from heterologous plasma; liver, gastric mucosa and other glands of guinea-pig have no

kinin-forming activity; and furthermore, accessory sex glands from rat, rabbit, cat and man have no kinin-forming activity.

Bradykinin in burns:

As bradykinin produces both vasodilatation and increased capillary permeability it may be one of the substances released in thermal damage. Rocha e Silva and Rosenthal (1961) found that histamine, bradykinin and adenosine compounds appeared in the fluid in an air pouch on the dorsum of rats, heated at 96°C for 15 seconds. The authors have suggested that the amount of bradykinin present follows that of histamine, but more evidence is necessary before this can be accepted; in any case the heating is very severe, as shown by the presence of adenyl compounds. The effect of various temperatures has also been studied in rat forelimbs perfused by a coaxial cannula (Rocha e Silva and Antonio, 1961). At 37°C the perfusate was inactive; at 43° - 45°C bradykinin-like activity appeared, but histamine appeared only at 57°C or above. The authors suggest that protease activation due to thermal damage acts on bradykininogen and releases bradykinin. Thermal damage has long been known to produce increased proteolytic activity. On the basis of evidence available so far, involvement of bradykinin in burns appears likely, but further work remains to be done.

Bradykinin in central nervous system activity:

Normal and pathological human cerebrospinal fluid has been investigated for smooth muscle stimulant activity, as well as for bradykinin-forming activity. Chapman and his co-workers (Chapman and Wolff, 1958, 1959; Chapman, Goodell and Wolff, 1959a, 1959b; Chapman, Ramos, Corrado, Forbes and Symes, 1960) reported that specimens from persons with no active lesion of the central nervous system had at most traces of oxytocic or bradykinin-forming activity. Specimens from patients with active inflammatory or degenerative conditions were usually active. They later found (1960) that when the sciatic nerve or brain of cat was electrically stimulated briefly, effluent from perfusate cerebral ventricle showed a transient increase in bradykinin-forming activity. The above-mentioned findings in human subjects could not be confirmed by Coppen and Lewis (1961) who found that enzymic activity, present in about one-quarter of samples studied were distributed randomly among patients with and without central nervous system disorders. Since these findings are in conflict, it should be noted that Chapman and his co-workers employed an unsatisfactory pharmacological test. They tested a fixed amount of cerebrospinal fluid, alone or after incubation with pseudo-globulin, and noted the presence or absence of contraction in rat uterus. All results were "negative", or "type I" or "type II", and all these contractions were small, as judged by the tracing illustrated. No attempt was made to express the amount of activity, in terms of a standard substance. In view of the very steep dose-response slope of bradykinin

on rat uterus, this kind of evaluation will greatly exaggerate any difference between "negative" or "type I or II" results, particularly if the negative results are due to quantities of bradykinin which are only slightly subthreshold.

Bradykinin and colostrum:

Urinary kallikrein, or calf saliva on incubation with bovine colostrum develops smooth muscle stimulant activity. The active principle, called colostrokinin is similar to plasma kinin (Guth, 1959, 1960). Plasma kinin is more akin to urine-colostrokinin than saliva-colostrokinin, as judged by parallel quantitative assay. Suckling by the newborn results in incubation of colostrum with saliva, presumably leading to formation of saliva-colostrokinin. It is postulated that this may facilitate the passage of protein from the neonatal gut to the blood (Guth, 1959), thus permitting the absorption of antibody gamma-globulin in the newborn. As colostrum is replaced by milk, the kinin-forming activity disappears.

Bradykinin in pregnancy and parturition:

Little information is available on this aspect. The amount of kinin formed by various activation procedures declines during labour, and is at its lowest at the end of the second stage of labour indicating an increased utilisation of bradykinin (Armstrong, Keele and Stewart, 1955). Human amniotic fluid has been shown to have bradykinin-like activity (Van Den Dreissche, 1960). Furthermore, in pregnant women kininase activity in serum is increased (Werle,

1960). Against speculation about involvement of bradykinin in pregnancy and labour, Berde and Saameli (1961) have pointed out that the oxytocic activity of bradykinin is much greater in vitro than in vivo. This might be explained by kininase activity. More information is required before the role of bradykinin in parturition can be evaluated.

Anaphylaxis:

Only one report on the appearance of bradykinin in anaphylaxis is available. Beraldo (1950) collected blood from sensitised dogs before and "several minutes" after shock, and tested it by direct application to the isolated guinea-pig ileum in presence of mepyramine. He reported "bradykinin activity" in 7 out of 18 experiments, and in some of these samples activity appeared only after incubation. This evidence and Beraldo's conclusions are discussed later in Part III ("Discussion").

Malaria:

Recently Tella and Maegraith (1962) have reported a decrease in the blood bradykininogen level in monkeys with experimental malaria. The actual amount of bradykinin involved is hard to assess because their method involved loss of bradykininogen during treatment prior to assay.

ANAPHYLAXIS:

The term anaphylaxis was coined by Portier and Richet (1902) to describe the characteristic and violent reactions in animals to substances, not necessarily toxic, to which they have previously been sensitised. The general picture of anaphylaxis varies from species to species, according to the tissue or organ which is most affected ("shock organ"), though changes in other tissues also contribute to the overall picture. For example, lung is the shock organ in the guinea-pig, and bronchoconstriction is the cardinal feature of anaphylaxis in this animal.

The steps leading from antigen-antibody reaction to these violent events is still not fully understood and various theories exist. The humoral theory was the earlier thesis to be put forward. It was known that when blood was treated with antigen-antibody precipitate in vitro, toxic products ("anaphylatoxin") were formed, which on injection into animals could reproduce the features of anaphylaxis. It was therefore postulated that antigen and antibody, combining in the blood stream would similarly lead to the formation of various toxic products and thus produce anaphylactic phenomena. Later findings, however, cast serious doubts on this *ideant*. Anaphylatoxin was shown to form when serum was treated with not only antigen-antibody precipitate, but such non-specific substances as peptone, agar and kieselguhr. The necessity of a "lag period" before injection of antibodies led to development of

passive sensitisation also went against the concept of the whole process taking place in the blood stream; in that event no lag period would have been necessary. The humoral theory as such gradually fell into disfavour and was displaced by the "cellular theory" for which there was increasing experimental support. This postulates that anaphylaxis occurs only when antigen combines with antibodies fixed on cells. If large amounts of antibody are present in circulation, it combines with antigen, thus preventing its union with sessile antibodies, and anaphylaxis does not occur. So the relative amounts of antibody in blood and tissue would decide whether an immune or anaphylactic response would follow challenge with antigen.

Enzymatic mechanisms in anaphylaxis:

Enzymic activity has long been suggested as the biochemical mechanism leading to the features of anaphylaxis. The earlier concept was that intracellular precipitation was followed by digestion with adsorbed protease, forming toxic substances like peptone. When non-precipitating antibodies were shown to produce sensitisation (Kabat and Ben$_a$cerraf, 1949; Kuhns and Pappenheimer, 1952) this theory became unrealistic. The nature of the enzymic mechanisms involved is still a subject of controversy.

Proteolysis:

The idea of involvement of a proteolytic enzyme is quite old, and followed the findings that administration of

peptone and other protein degradation products could mimic anaphylactic phenomena (Vaughan and Wheeler, 1907; Biedl and Kraus, 1909). The earlier theories suggested that antigen-antibody precipitate adsorbed protease which acted on it to release "anaphylatoxin" (Friedberger, 1909, 1910; Friedmann, 1909). It was found later that addition of a large amount of non-specific substances could form anaphylatoxin. This was interpreted to indicate that a delicate balance normally exists between proteolytic and anti-proteolytic activity in blood, which can be easily upset. Bronfenbrenner (1915) and Jobling and Petersen (1914) suggested that anaphylaxis alters this balance, probably by reducing the antiprotease activity; this leads to a heightened protease activity which in turn produces the features of anaphylaxis.

It was later found that addition of antigen produces contraction of isolated sensitised guinea-pig uterus and ileum, washed virtually free of blood (Schultz, 1910; Dale, 1913). This meant that anaphylactic phenomena could take place in the virtual absence of blood or serum. The serum activation theory was thus gradually abandoned and replaced by the cellular theory with histamine as the central causative agent.

Interest in proteolytic enzymes was revived when Rocha e Silva (1939, 1940) showed that intravenous injection of trypsin could mimic anaphylactic phenomena. He reported (Rocha e Silva, 1943) that compounds of histamine with amino-acids were pharmacologically inactive, but activity appeared

on hydrolysis, and suggested that histamine was possibly bound to free carboxyl groups of proteins by peptide linkages. It has been argued, however, that cold trichloroacetic acid and other mild hydrolysing agents, which do not split peptides *in vitro*, do release histamine *in vivo* (Schayer, 1956; McIntire, 1956).

The modern version of the protease activation theory is that antigen-antibody reaction activates normally inactive proteases present in tissue or serum or both. The enzymes then damage certain specified cells to release histamine, 5-hydroxytryptamine, heparin etc., which produce the observed response. In addition, the liberated enzyme may directly attack vascular endothelium, causing further damage (Burdon, 1952).

Further reports on protease activity in anaphylaxis followed, both *in vivo* and *in vitro*. Ungar and Mist (1949) had reported that when antigen was added *in vitro* to cell-free sensitised guinea-pig plasma or serum, protease activity was increased. Similar reports have been made regarding the blood of rabbit and dog (Geiger, 1952; Cliffton, 1952). These results were contested by MacIntire, Roth and Sproull (1950) who used rabbits. Working *in vitro*, Ungar and Damgaard (1955) found that on challenge of sensitised guinea-pig lung, increase in protease activity and histamine release followed a parallel course. Since protease inhibitors like soya bean inhibitor suppressed histamine release, they suggested that proteolysis was the basic event which led to histamine

release. Hayashi (1956) found increased protease activity on addition of antigen *in vitro* to a culture of monocyte cells from rabbit peritoneum.

In vivo, Lowell, Franklin, Schiller and Follensby (1956) have reported raised protease activity in human blood during attacks of hay fever. Increased protease activity has been reported in the blood, urine (Damgaard and Ungar, 1952) and lung (Herberts, 1949, 1955a, 1955b, 1958) of guinea-pig.

Some important evidence is now available regarding the nature of the enzymes involved in the release of histamine during anaphylaxis in guinea-pig lung (Austen and Brocklehurst, 1961a). These workers found that ester and peptide substrates and several types of the inhibitors of chymotrypsin, but not those of trypsin, carboxypeptidase or leucine aminopeptidase, inhibited the release of histamine. Their results suggest that these substances inhibit an antigen-antibody activated step because they had no activity if applied beforehand but washed out of the tissue before challenge with antigen. These workers have suggested that activation of a chymotrypsin-like enzyme is necessary for anaphylactic release of histamine from guinea-pig lung. In this connection, it is interesting to note that rat mast cells have been reported to contain trypsin-like (Glenner and Cohen, 1960), chymotrypsin-like (Benditt and Arase, 1959) and leucine aminopeptidase-like (Braun-Falco and Salfeld, 1959) enzymes.

While evidence is available on the association of anaphylaxis and activation of enzymes, its interpretation is still a matter of controversy. Tissue contains a number of proteases and other enzymes, and little is known about most of them. Part of the confusion in this field is caused by equating fibrinolytic or plasmin-like activity with protease activity. Proteases vary widely in their substrate specificity, and lack of activity on fibrin, casein or haemoglobin does not necessarily indicate lack of protease activity; it may merely mean that the appropriate substrate had not been employed. Furthermore, if an active substance has a lytic effect on a particular substrate (e.g. fibrin) it does not necessarily follow that it is identical with any one particular enzyme (e.g. plasmin). It should also be remembered that blood contains a number of proteases; some exist as precursors, some are combined with inactivators. One has only to consider the many ways in which clotting can take place to illustrate this (Albrechtsen, 1959).

Non-proteolytic enzyme:

Mongar and Schild (1957a, 1957b, 1958) investigated the effect of various enzyme inhibitors, oxygen lack, calcium lack and other factors on the release <u>in vitro</u> of histamine from anaphylactic guinea-pig lung. On the basis of their findings, they have put forward the hypothesis of an intracellular, heat-labile, oxygen-requiring enzyme system which is responsible for the release of histamine

and perhaps other pharmacologically active substances. They suggested that both free sulphydryl groups and disulphide groups are necessary for the anaphylactic reaction (Edman, Mongar and Schild, 1961; Mongar and Schild, 1962). Protein denaturants inactivated the mechanism. They found that oxygen lack depressed histamine release. The effect of temperature on the lung tissue preparation was in keeping with enzymic activation. Mongar and Schild (1957) found that calcium was an essential ion for histamine release. The effect of pH-alteration also resembled an enzyme-pH curve. In the opinion of Mongar and Schild (1957a, 1957b, 1958) the postulated enzyme system is not proteolytic, though protease activation and histamine release could be parallel phenomena. They have pointed out that heating above 45°C inactivates the anaphylactic mechanism, but not the proteolytic mechanism. Inhibition of anaphylactic histamine release, whether by heat or by chemical inhibitors, is accompanied by a potentiation of histamine release by organic bases, yet the action of both types of releasers lead to proteolysis. These authors suggest that proteolysis is a general feature accompanying histamine release due to different causes, while the various inhibitors they have tested act on a more specific mechanism found so far to operate only in anaphylaxis.

Lecithinase-A hypothesis:

Uvnäs and Thon (1961) have suggested that lecithinase is normally present in an inactive form on the mast cell surface. Compound 48/80 and antigen activate lecithinase and consequently mast cells are disrupted and

histamine is released. This hypothesis was based on their findings that lecithinase disrupts rat mast cells. This phenomenon, in common with disruption by compound 48/80 or antigen, is inhibited by sulphydryl-blocking agents. Other lecithinase-containing agents like snake venom and bee venom also release histamine. Furthermore, calcium lack inhibits lecithinase activity in vitro (Long and Penny, 1957) the disruption of mast cells by lecithinase activity or antigen challenge in vitro. In view of the suggested lipid nature of SRS (Chakravarty and Uvnäs, 1960), a mediator in anaphylaxis, this postulation is of much potential interest, but the evidence available in support so far is not adequate. It is possible, of course, that more than one enzyme system is involved in the liberation of different mediators of anaphylaxis (Brocklehurst, 1962).

Pharmacologically active substances in anaphylaxis:

The anaphylactic response is brought about by pharmacologically active substances which are either released or formed in tissue as a result of antigen-antibody reaction. Menkin has applied a similar concept with respect to tissue reactions in inflammation. Of the mediators in anaphylaxis, more information is available about histamine than any other. In recent years, information has accumulated about SRS-A, 5-hydroxy-tryptamine and heparin. A number of other substances have also been proposed, but sufficient evidence is not yet available.

Histamine:

The possible involvement of histamine as a mediator in anaphylaxis was realised when Dale and Laidlaw (1910) pointed out that the toxic features of histamine could mimic anaphylactic phenomena to a large extent. This was soon confirmed by others (Biedl and Kraus, 1909, 1911; Aronson, 1912). During 1927-29, the view that antigen-antibody reaction releases histamine from tissues, of which it is a natural constituent, was clearly formulated. It was subsequently shown that histamine is released following antigen-antibody reaction in most species - dog, guinea-pig, rabbit, cat, cockerel and monkey (Feldberg, 1961). This occurs after challenge both in vivo and in vitro. In man, histamine is released during attacks of allergy, and on challenge in vitro of isolated perfused human bronchial chain from allergic patients (Schild, Hawkins, Mongar and Herxheimer, 1951). Concerning rats, there have been contradictory reports. Mota (1957) has reported raised blood histamine levels. Isolated tissue fragments of sensitised skin and mesentery (Mota and Ishii, 1960) have been shown to release histamine; disruptive changes, such as occur with histamine liberators have also been reported (Mota and Ishii, 1960) in vitro. Brocklehurst (1960) did not find any significant histamine release due to anaphylactic shock in rat lung, liver or hindquarters; nor could the anaphylactic changes in rat mast cells be confirmed by Sanyal and West (1958).

The release of histamine has been taken to indicate

the degree of tissue response to antigen-antibody union and much work done to elucidate the chain of events leading therefrom to histamine release. A résumé of the contending theories has been already given under "enzymic processes in anaphylaxis".

The role of histamine as the major mediator in most species can now be taken as well-established. As Feldberg (1961) has pointed out, the trend is now in the opposite direction - i.e. to explore phenomena which cannot be satisfactorily explained by histamine release. The search for other mediators has been fruitful in recent years, and good evidence has been obtained about SRS-A, 5-hydroxytryptamine and heparin as mediators, apart from inconclusive evidence about other proposed mediators.

SRS-A:

Kellaway and Trethewie (1938) had found that in perfused anaphylactic guinea-pig lung, a substance appeared which produced a slow contraction, thus modifying the relaxation phase of the quick contraction of histamine, on guinea-pig ileum. In 1938, they had found this substance in effluent from lung and liver perfused with snake venom. In view of the slow contraction it induced, it was termed SRS (Slow Reacting Substance). Brocklehurst (1953 et seq.) found that during anaphylaxis in guinea-pig lung in vitro, a smooth muscle contracting, mepyramine-resistant, slow reacting substance appeared in the effluent. He established the separate identity and pharmacological

profile of this substance, and, pending identification of its chemical nature, named it SRS-A (Slow Reacting Substance of Anaphylaxis) to distinguish it from other possible slow reacting substances, not necessarily identical. SRS-A could be distinguished from histamine, acetylcholine, 5-hydroxytryptamine, bradykinin, substance P, adenosine triphosphate and other substances. It was found in anaphylactic lung perfusate of rabbit, monkey and allergic man also, though not from rat. SRS-A was not found to be formed from platelets and other constituents of blood; it originates from lung tissue. Unlike histamine, it is not released from preformed stores, but formed as a result of antigen-antibody reaction in the lung. In guinea-pig the substance was found to occur chiefly in the lung and vascular tissue. Chemical identification of this substance has been rendered difficult by its instability after purification: Brocklehurst (1960) has suggested that it could be a lipopolysaccharide. Recently Smith (1962) has suggested that it is very much akin to methoxyneuraminic acid, but further proof is required before this can be accepted. The intimate mechanism of its release has been a matter of controversy. Austen and Brocklehurst (1961) are of the opinion that the chain of events leading from antigen-antibody reaction to the release of histamine and SRS-A must have an early part in common, but the final stages are different as shown by the different rates at which histamine and SRS-A leave shocked lung. Chakravarty (1959) has suggested the same mechanism for the release of

both. The role of mast cells as a source of SRS-A has been a matter of controversy. A good correlation between the amounts of histamine and released SRS-A would go in favour of mast cells as the major source of SRS-A. This is difficult to show because of biological variation inherent in such experiments, e.g. different levels of sensitivity, of tissue content of histamine and SRS-precursor, and different rates of diffusion and inactivation of the enzymes and pharmacological agents involved. Chakravarty (1960) has claimed such a correlation, but his use of litter-mate animals introduce a bias in favour of such correlation. Boréus and Chakravarty (1960) have also reported a correlation between numbers of mast cells involved, and amounts of histamine and SRS released. Brocklehurst (1962) considers the correlation too poor to support such a theory; he has also pointed out that in vitro shock of guinea-pig mesentery leads to release of histamine but not SRS-A, and so mast cells are unlikely to be a major source of SRS-A. Until a suitable and specific antagonist for SRS-A is available, evaluation of its role in anaphylaxis will not be complete. However, it has explained a number of anaphylactic phenomena which could not be explained with histamine alone. Furthermore, it is a very likely candidate for the causation of asthmatic bronchoconstriction, a probability of great clinical importance.

5-hydroxytryptamine:

In recent years, this substance has been investigated

with regard to its possible role as a mediator in anaphylaxis. The mast cells of rat and mouse contain 5-hydroxytryptamine (Benditt, Wong, Arase and Roper, 1955). The platelets of rabbit are rich in 5-hydroxytryptamine, but those of man, rat or guinea-pig are not (Humphrey and Jaques, 1954, 1955). 5-hydroxytryptamine occurs also in certain parts of the gastro-intestinal tract and the central nervous system.

5-hydroxytryptamine does not seem to be a mediator in anaphylaxis in man (Brocklehurst, 1958), dog (Sanyal and West, 1958) and guinea-pig (Herxheimer, 1955; Fink and Gardiner, 1956; Wiessbach, Waalkes and Udenfriend, 1957; Brocklehurst, 1958a), though Sanyal and West (1958) reported the release of a rat uterus contractile substance from guinea-pig lung, antagonised by lysergic acid diethylamide. Alberty and Schiede (1953) have reported somewhat similar findings. Hawkins and Rosa (1959) reinvestigated earlier reports of Campbell and Nicoll (1940), and found that release of such oxytocic activity was not necessarily confined to anaphylaxis, and could be induced by the squeezing effect of histamine bronchoconstriction. In the rabbit it has been found that the 5-hydroxytryptamine content of anaphylactic plasma rises in the circulating blood, but the content in whole blood decreases: this is probably due to the trapping and destruction of platelets in the pulmonary vascular bed; the pulmonary 5-hydroxy-tryptamine content is found to rise (Sanyal and West, 1958; Waalkes and Coburn, 1959). The free 5-hydroxytryptamine

found in plasma is probably of platelet origin. The role of 5-hydroxytryptamine in anaphylaxis in rats and mice is contested. While it plays a major role in anaphylactoid oedema in rat (Rowley and Benditt, 1956; Parratt and West, 1956, 1957, 1958; Doepffner and Cerletti, 1958), it plays at best only an insignificant role in the increased vascular permeability of active and passive cutaneous anaphylaxis (Inderbitzin and Craps, 1957; Sanyal and West, 1958; Brocklehurst, Humphrey and Perry, 1960). Weissbach, Waalkes and Udenfriend (1957), however, suggest that since comparatively large amounts of the amine, its synthesizing and destroying enzyme are present in rat lung, it may have a role to play. The role of 5-hydroxytryptamine in mice also has been contested. It has been shown (Fink, 1956) that antigenic contraction of sensitised isolated mouse uterus can be suppressed by 5-hydroxytryptamine antagonists. Weiser (1957) and Fox, Einbinder and Nelson (1958) have reported that lysergic acid diethylamide and reserpine prevent anaphylactic features in mice mainly attributed to histamine.

On the basis of evidence so far available, 5-hydroxytryptamine plays some part during anaphylaxis in rabbit, but adequate evidence is not available to ascribe a role firmly to it in other species.

Heparin:

Heparin is another pharmacologically active substance

occurring in mast cells which is known to be liberated in anaphylaxis. It was reported to occur in mast cells as early as 1937 (Jorpes, Holmgren and Wielander, 1937). MacIntosh and Paton (1957) believe that basic histamine in mast cells is balanced in charge by the acid heparin which explains why the two substances are often released together. Eagle, Johnstone and Ravdin (1937) first suggested that incoagulability of dog blood during anaphylaxis was due to the release of heparin from liver into blood. Jaques and Walters (1941) established this view and showed that mast cells were the site of origin in the liver. Other workers extended these findings to isolated perfused liver later (Rocha e Silva, Scroggie, Fidlar and Jaques, 1949; Scroggie and Jaques, 1949). However, incoagulability of blood does not occur in rabbit or guinea-pig; the coagulation time is slightly prolonged. A metachromatic substance, related to heparin but not an anticoagulant, appears in blood. Like heparin, this appears to be released together with histamine (Monkhouse, Fidlar and Barlow, 1952).

Bradykinin:

Only one report has been published so far on the possible activation of bradykinin in anaphylaxis. Beraldo (1950) found that the blood of dogs in anaphylaxis, when added to a bath containing an isolated guinea-pig ileum could produce a slow contraction in the presence of atropine and mepyramine. He concluded that this was due to increased bradykinin content of blood. The extent of

such increase was found to be quite variable, and some post-shock samples needed incubation before any activity could be detected: hence he felt that bradykinin was unlikely to play any important part in anaphylactic response. At that time it was not known that dilution of blood in physiological solution, or its exposure to glass leads to formation of bradykinin (Schachter, 1956; Armstrong, Jepson, Keele and Stewart, 1957) and appropriate precautions against activation during experimental manipulations were presumably not taken. This makes it difficult to interpret Beraldo's findings. A detailed discussion and evaluation of these findings is given later in Part III under "Discussion".

Substance of Campbell and Nicoll:

In 1936, Ungar and Parrot found that challenge of sensitised tissue in an organ bath produced a contraction in another suitable unsensitised smooth muscle preparation mounted in the same bath. Employing this technique, Campbell and Nicoll (1940) found that during anaphylaxis, a substance was released from guinea-pig lung which contracted rat uterus <u>in vitro</u>. Further information is not available about the possible nature of this active principle. These workers do not seem to have employed lungs washed free of blood, and it is possible that a certain amount of blood was present in the preparation. Hawkins and Rosa (1959) repeated and interpreted this work as mentioned earlier.

PART II

E X P E R I M E N T A L

METHODS AND MATERIAL

METHODS AND MATERIAL

PREPARATION OF ANTIGEN:

Except where mentioned, egg albumin was used as the antigen throughout the present experiments.

Crude egg albumin:

A freshly-made 10% solution of egg albumin in normal saline containing 0.5% phenol was used. After phenol was added, the mixture was left at room temperature for an hour and any precipitate discarded.

Crystalline egg albumin:

1% solution in normal saline was used. This was stored at -10°C.

Alum-adsorbed egg albumin:

4.6 ml of N sodium bicarbonate was added to 10 ml of 10% solution of crude egg albumin in normal saline. A 10% aqueous solution of potash alum was added to it dropwise while stirring, until the pH came to 6.8 (B.D.H. capillator). Evolution of CO_2 ceased as this point approached. The mixture was kept at 4°C for 2-3 hours and centrifuged. The precipitate was washed twice in distilled water, and finally was suspended in 10 ml phosphate buffer at pH 7.4, and stored at 4°C.

Freund's adjuvant antigen:

The emulsion had the composition recommended by Dresser (1960), as given below.

Light liquid paraffin	2 ml
Heat killed Mycobacterium tuberculosis	4 mg
Sorbitol mono-oleate (Crill K 16)	1 ml
10% egg albumin in normal saline	2 ml

The solution was squirted through the nozzle of a tuberculin syringe until a suitable emulsion was obtained; this did not spread when a drop was placed on the surface of distilled water. The emulsion was usually used the same day, but occasionally stored overnight at 4°C. It was warmed to body temperature just before use, and a wide bore hypodermic needle was used for injection.

SENSITISATION OF ANIMALS:

Guinea-pigs:

Smooth-haired guinea-pigs of either sex about 3 weeks old and approximately of 200 g bodyweight were injected with 1 ml crude egg albumin solution intraperitoneally and 1 ml subcutaneously. The animals were used 21 to 35 days later. After four weeks, 2 mg alum-adsorbed antigen in 3 ml normal saline was injected intraperitoneally as a booster once every 2-3 weeks. These animals were used 15 to 28 days after the last of such injections, which maintained sensitisation at a high level.

Rabbits:

Rabbits of either sex of 1.5 to 2.5 kg bodyweight were used. 50 mg of antigen in Freund's adjuvant was injected subcutaneously and repeated after one week. Six weeks later, six intravenous injections of alum-precipitated antigen were given at intervals of three days: these were graded, rising from 2 mg to 10 mg. The animals were employed six to eight days after the last injection.

Rats:

Albino male rats (Wistar type) of 100 to 150 g bodyweight were used. 25 mg of Freund's adjuvant antigen was injected subcutaneously and repeated one week later. After three weeks, 2 mg antigen was injected intravenously. The animals were used two to three weeks later.

COLLECTION OF BLOOD:

Guinea-pigs and rats were anaesthetised with urethane (1.75 g/kg bodyweight) given partly intraperitoneally and partly subcutaneously. A polythene cannula was tied into one common carotid artery for the purpose of bleeding and another was tied into the external jugular vein for administration of antigen. In the case of rabbits, intravenous anaesthesia was induced with sodium pentobarbitone and antigen was injected through the marginal ear veins.

BIOASSAY METHODS:

Rat uterus:

Virgin albino rats were brought into artificial oestrous by a subcutaneous injection of stilboesterol (10 µg/100 g bodyweight) in arachis oil: 18-20 hours later, the animal was killed, and a section of the uterine horn, about 2 cm long, was suspended in a 1 or 2 ml organ bath containing oxygenated de Jalon's solution at 30°-31°C. The de Jalon solution contained 10^{-6} g/ml atropine sulphate, and when 5-hydroxytryptamine was to be excluded, freshly made 2-bromo-lysergic acid, 2×10^{-7} or 5×10^{-7} g/ml. Bradykinin was usually left in the bath for 60 seconds, though in some preparations this had to be longer. A cycle of 5 minutes was usually employed. The writing lever exerted a load of 300-400 mg and gave a magnification of 4 to 6. 2 + 2 point assays were done only when determining the potency of crude bradykinin made from ox plasma in terms of synthetic material or Rocha e Silva's Pool I standard, kindly given by Professor H.O. Schild of University College, London. Otherwise, in view of the comparatively long time-cycle and the nature of the experiments, a simple bracketing assay was employed (Dale, 1912). Such an assay is shown in figure 1. The kymograph was stopped between contractions in this as in most other assay experiments.

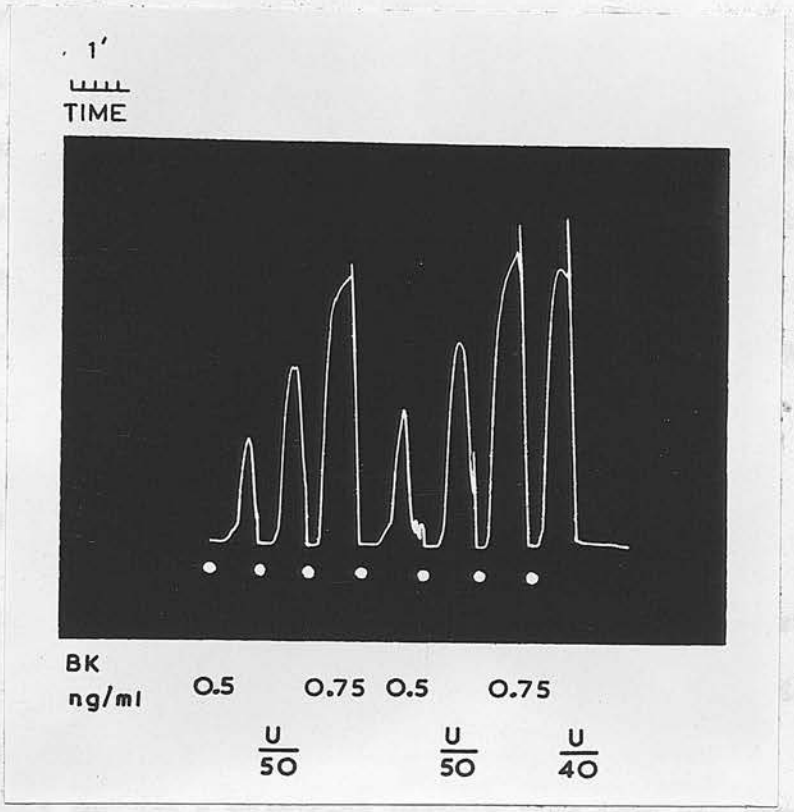


Fig. 1: Rat uterus: Bracketing assay of extract of anaphylactic sample of guinea-pig blood (= U) against bradykinin (= BK).

Guinea-pig ileum:

A piece of terminal guinea-pig ileum about 2 cm long, was put up in a 1-2 ml bath at 34° to 35°C in oxygenated Tyrode solution. An exposure of 30 to 45 seconds and a cycle of 4 minutes was generally adequate.

Rat duodenum:

The proximal 2-3 cm of duodenum from albino rats of 120-150 g bodyweight was suspended in de Jalon solution at 30°-31°C in an organ bath. The tissue had been stored at

4°C for two or three hours previously. The lever used exerted a tension of 500 mg on the tissue and magnified the response 15-20 times. It was found that results were better, i.e. the resting tonus was more constant, if the tissue was under constant wash by slow overflow. In these experiments, the overflow was stopped for 20 to 30 seconds before bradykinin was added to the bath.

RAT BLOOD PRESSURE EXPERIMENTS:

Albino rats of 150 to 250 g bodyweight were anaesthetised with sodium barbitone 0.5 g/kg and urethane 0.6 g/kg intraperitoneally (Amin, 1953). Blood pressure was recorded through a polythene cannula tied into one common carotid artery, and leading to a Condon single-limb mercury manometer. A polythene cannula in the external jugular vein was used for administration of drugs or antigen dissolved in 0.2 ml saline.

ISOLATED LUNG PERFUSION:

This was done according to the method desribed by Brocklehurst (1960). The guinea-pig was killed by dislocation of the neck; the trachea was at once ligated and the lungs with the heart attached were dissected out without handling them. An incision was made into the right ventricle: through this, a glass-tipped flexible cannula was tied into the pulmonary artery, so that the tip was proximal to the bifurcation of the pulmonary artery.

A similar cannula was tied into the left auricle. The preparation was suspended in a water-jacketed chamber at 37°C, with the lungs inflated through a tracheal cannula to about two-thirds of the in vivo volume. Tyrode solution, prewarmed to 37°C, entered through the pulmonary artery cannula, the effluent being collected through the left auricular cannula. Blood was flushed out by an initial flow of about 20 ml/min, and the rate was then reduced to about 2 ml/min. Collection started only after the perfusate appeared to be free from blood. Collected perfusate was kept on ice till tested on the same day.

PREPARATION OF DOG PSEUDOGLOBULIN SUBSTRATE:

The method is essentially that of Holdstock et al. (1957) and Horton (1958). Healthy adult mongrel dogs were anaesthetised with intravenous thiopentone sodium, and bled through a polythene cannula tied into the common carotid artery. The blood was collected into a polythene bottle, containing heparin in a final concentration of 1 I.U. per ml. The blood was centrifuged at approximately 900 g for 15 minutes to separate plasma, which was then kept at 56° to 58°C for three hours. The plasma was then subjected to dialysis within a viscose cellophane tube (Visking Corporation) at 4°C against 10-20 volumes of distilled water for 36 hours, during which the water was changed three times. The plasma was treated with ammonium sulphate, and the fractional precipitate between 33% and 46% saturation was separated. The precipitate was dissolved in 4.5% NaCl solution, and

dialysed overnight at room temperature against running tap water. The solution was taken to pH 2 with hydrochloric acid, incubated at 37°C for 15 minutes, and then brought back to pH 7.4. The material was distributed in ampoules, freeze-dried, sealed in dry air and stored at -10°C until used.

PREPARATION OF CRUDE BRADYKININ:

During the earlier part of the investigation, crude bradykinin was used for bioassay purposes. Defibrinated ox blood was obtained from the abattoir, and after treatment as shown above, the material was suspended in phosphate buffer pH 7.4. This was incubated with trypsin (0.4 mg/ml plasma) at 37°C for 20 minutes after which 2 volumes of hot ethanol was added to the reaction mixture. The mixture was kept in a boiling water bath for five minutes, cooled, and dried at 35°C in partial vacuum in the presence of a slow stream of nitrogen. The dried material was stored at -10°C.

MATERIAL:

Analar grade reagents or the best available were used throughout. All solvents were redistilled in glass. Water was glass-distilled or deionised in "Elgastat". Crude egg albumin (British Drug House), twice-crystallised egg albumin (L. Light & Co.), Crill K 16 (Croda Ltd., Goole, Yorks), Mycobacterium tuberculosis residue from P.P.D.

preparation (Veterinary Research Laboratory, New Hall, Weybridge, Surrey), magnesium-free trypsin (Armour), salt-free chymotrypsin (Armour), soya bean trypsin inhibitor, and hexadimethrine bromide (Abbott) were obtained commercially. Synthetic bradykinin (Parke Davis & Co.), 2-bromo-lysergic acid (Sandoz Ltd.) and mepyramine maleate (May and Baker Ltd.) were made available by the manufacturers.

During some of the earlier experiments, before synthetic bradykinin was obtained, crude bradykinin prepared as above was used for bioassay. This had been quantitated against a standard bradykinin sample of Prof. M. Rocha e Silva. Later, synthetic bradykinin was used. All amounts of bradykinin mentioned in the present work are in terms of the synthetic material.

Ethanol was redistilled in glass before use. Siliconed glassware or polythene was used throughout for the handling of blood or plasma.

RESULTS

SECTION I

DEVELOPMENT OF METHOD FOR ESTIMATION OF BRADYKININ IN BLOOD

SECTION I

ESTIMATION OF BRADYKININ IN BLOOD:

The study of a possible release of bradykinin in blood from its precursor globulin *in vitro* requires a technique which will neither form nor destroy bradykinin. As no suitable method was available, experiments described in this section were undertaken to develop a suitable method for estimation of bradykinin in blood.

Guinea-pigs, rabbits and rats were bled via a polythene cannula from the common carotid artery. Blood was permitted to flow out when required, straight into 4 volumes of glass-distilled ethanol at 2°-4°C. The volume of blood collected was found by directly reading off from a graduated glass cylinder, or by a graduated polythene centrifuge tube. As the blood flowed out, the blood-ethanol mixture was gently shaken to avoid, as far as possible, formation of large coagula. After collection, the mixture was thoroughly shaken to break up any lumps, and then centrifuged at 0°-4°C at approximately 3,500 g for 20 minutes. The supernatant, a clear fluid which often had a pale yellow tinge, was dried under reduced pressure at 35°-40°C in a slow stream of nitrogen. The dried material was then reconstituted with saline or de Jalon solution, usually to the original volume of blood. The reconstituted solution was assayed on rat uterus in the presence of atropine sulphate, 5×10^{-7} g/ml, and 2-bromo-

lysergic acid 2 to 5 x 10^{-7} g/ml.

Recovery tests:

The efficiency of this procedure was tested by the estimation of amounts recovered from known amounts of bradykinin added to blood. One aliquot of the sample of blood (control) was estimated for natural bradykinin content. In the test sample, the added saline solution of bradykinin was allowed to drip slowly into the cold ethanol out of a siliconed pipette held in such a way that as much as possible of the bradykinin came in contact with the blood as it ran from the arterial cannula before reaching the ethanol. In some experiments mixture was ensured by passing the blood and bradykinin together through a polythene tube flared into the shape of a small funnel. Samples to which bradykinin had been added for subsequent recovery both followed and preceded the control sample. In some experiments, the blood was collected in polythene or siliconed glass test tubes, and the appropriate amount withdrawn with a siliconed glass pipette. All samples were estimated for bradykinin as described above. The results with guinea-pig blood, rat blood and rabbit blood are set out in Tables 1, 2 and 3. Some entries in these tables show control values as < 3 ng etc. The estimated amount in that sample was less than the lowest limit detectable under the circumstances of that particular assay, e.g. 3 ng/ml. Thus the real control value in these cases may be anywhere between zero and the limit of detection. This experimental limitation tends to minimize the increase

Bradykinin in ng/ml blood					
Control	Added	Apparent recovery	Recovery minus control	Absolute loss	Recovery %
Directly taken into ethanol					
<4.6	32.9	31.6	27.0	5.9	82%
<3.4	57.5	50.7	47.3	10.2	82%
<5.7	11.5	12.4	6.7	4.8	58%
<5.7	131.4	97.6	91.9	39.5	69%
Collected in polythene					
13.8	131.4	101.6	87.8	43.6	76%
25.2	23.0	42.6	17.4	5.6	74%
15.9	38.3	46.7	30.8	7.5	80%

Table 1: Recovery of bradykinin added to guinea-pig blood

Bradykinin in ng/ml blood					
Control	Added	Apparent recovery	Recovery minus control	Absolute loss	Recovery %
11.0	15.0	24.9	13.9	1.1	92.3 %
7.5	30.0	37.0	29.5	0.5	98.3 %
7.0	45.0	32.4	25.4	19.6	56.4 %
6.0	60.0	50.0	44.0	16.0	73.2 %
7.8	33.3	32.4	24.6	8.7	74.0 %
Mean recovery: 78.8 S.E. 7.5 %					

Table 2: Recovery of bradykinin added to rat blood

Bradykinin ng/ml blood					
Control	Added	Apparent Recovery	Recovery minus control	Absolute loss	Recovery %
< 1.5	20	15.5	14.0	6.0	70 %
3.0	30	29.4	26.4	4.6	88 %
4.1	25	26.5	22.4	2.6	89%
6.4	33.3	38.3	31.9	1.4	96%
Mean recovery rate: 85.65 S.E. 5.49 %					

Table 3: Recovery of bradykinin added to rabbit blood

in bradykinin levels in active anaphylactic samples, and any bias so introduced will operate against the conclusion that there is a real difference between the control and experimental samples, which the results nevertheless show quite clearly.

The recovery levels in all three species appeared to be about three-quarters of the amount added. In the guinea-pig, polythene-collected blood tended to give a higher control value than ethanol-collected blood, but recoveries in both groups were of a similar order. The amount of bradykinin lost in the experimental procedure did not appear to be an absolute quantity, neither was it closely related to the amount added. It may represent the quantity trapped within the clot.

POTASSIUM CONTENT OF EXTRACTED BLOOD:

Since whole blood instead of plasma had been employed for ethanol extraction, K^+ values were estimated by flame photometry in extracts from blood of guinea-pig, rat and rabbit. In guinea-pig blood extracts, the mean K^+ content per ml blood extracted was 19.8 µEq (range 14-21); in rat blood extract, the mean content was 17 µEq (range 8-26); in rabbit, the mean K^+ content was 23.4 µEq/ml (range 15-35). The threshold concentration of K^+ producing contractions in rat uterus was found to vary between 4 and 8 µEq per ml bath fluid. Therefore, during assay on rat uterus, all samples were diluted at least fivefold before testing. However, the quick contraction due to K was quite

easily distinguished from the much slower contraction induced by bradykinin, and no serious potentiation was seen. This limitation of the degree of dilution imposes a lower limit of detection of bradykinin in blood by this method, notably in small samples. Since active samples needed far greater dilution than the minimum, there was no problem in the assay of those samples.

EFFECT OF BRADYKININ DEGRADATION PRODUCTS ON SMOOTH MUSCLE CONTRACTION PRODUCED BY BRADYKININ:

In view of the potentiation which cysteine exerts on the smooth muscle contraction produced by bradykinin (Picarelli, Henriques and Oliveira, 1962) it was necessary to investigate whether bradykinin split products could interfere with the assay of bradykinin. Bradykinin was incubated for one hour at 37°C with salt-free chymotrypsin (0.25 mg/µg bradykinin). At the end of the incubation, the mixture was boiled for five minutes, and the residual amount of bradykinin estimated; this was 5% or less of the original amount. Addition of this degradation product to bradykinin did not produce any potentiation, as tested on isolated rat uterus and guinea-pig ileum, which could not be satisfactorily explained by the residual amounts of bradykinin in the digestion mixture. This is shown in figures 2 and 3.

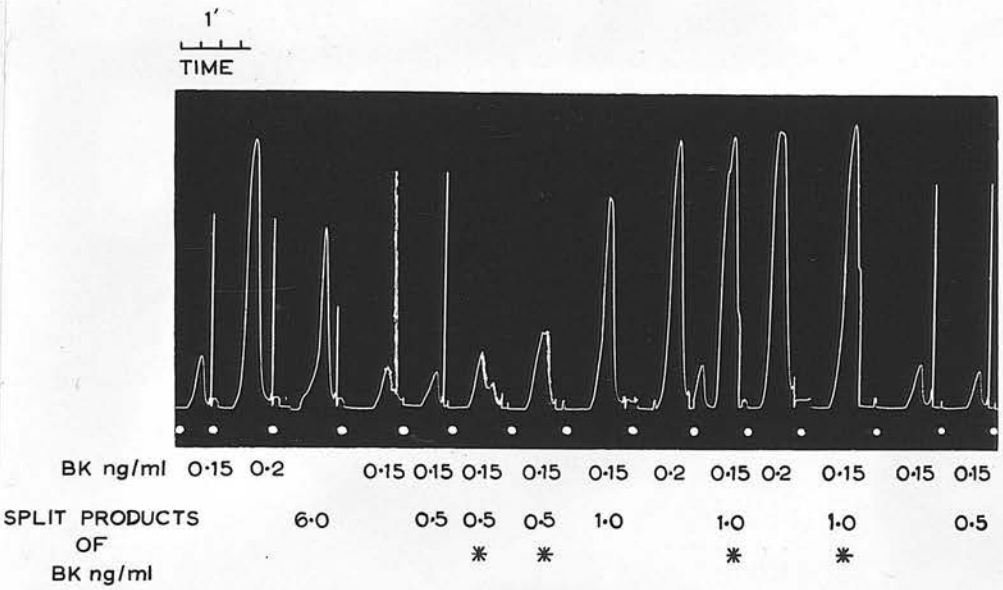


Fig. 2: Effect of bradykinin (= BK) split products on bradykinin-induced contractions of rat uterus; asterisk (*) denotes that bradykinin split products were added 60 seconds before bradykinin. Note the residual oxytocic activity of BK split products, enough to explain apparent potentiation of contraction.

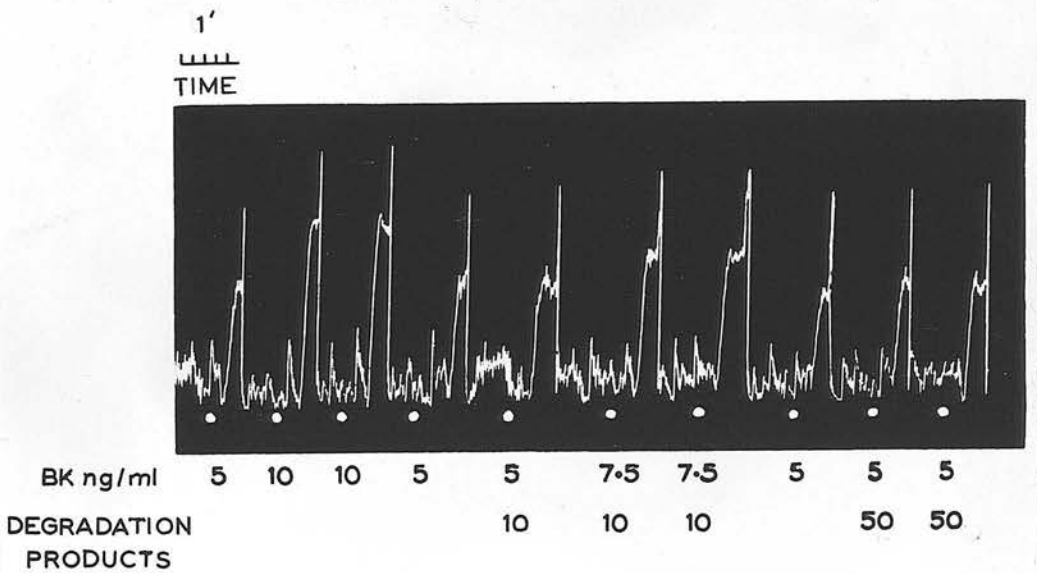


Fig. 3: Effect of bradykinin (= BK) split products on bradykinin-induced contraction of guinea-pig ileum.

To summarise, the experiments described in this section led to an ethanol-extraction method for estimation of bradykinin content of blood, avoiding spontaneous formation and destruction during experimental manipulation as far as possible. The assay of bradykinin is not seriously affected by the presence of extracted or extraneous material in the samples.

SECTION II

RISE IN BLOOD BRADYKININ LEVEL DURING ANAPHYLAXIS AND INDIRECT EVIDENCE FROM RAT BLOOD PRESSURE EXPERIMENTS

BLOOD BRADYKININ LEVELS IN ANAPHYLAXIS:

The experiments described in this section were designed to investigate (a) whether bradykinin level in blood rose after anaphylaxis, and if so, (b) what time course was followed. Sensitised guinea-pigs, rats and rabbits were used.

GUINEA-PIGS:

Intraperitoneal challenge:

2 mg alum-adsorbed antigen suspended in 3 ml 0.9% NaCl was injected intraperitoneally. By this means, the antigen is only slowly absorbed and shock is prolonged and thus not fatal. The animals were bled, and blood bradykinin level determined at $\frac{1}{2}$, 1, $1\frac{1}{2}$ and 2 hours after challenge. In the control animals 3 ml normal saline was injected intraperitoneally $\frac{1}{2}$, 1, $1\frac{1}{2}$ or 2 hours before bleeding. The blood bradykinin levels in control and shocked guinea-pigs is shown in table 4 and figure 4 illustrates the time-course.

Each figure in table 4 represents a different animal; only one sample was drawn from any one animal in case this procedure itself should cause a rise in blood bradykinin. No definite evidence is yet available about a rise in blood bradykinin following haemorrhagic shock, but

it seems probable.

Bradykinin ng/ml blood				
Time in hours				
Control	0.5	1.0	1.5	2.0
< 3.6	11.4	40.9	21.7	14.5
< 1.8	18.1	31.4	19.3	5.5
< 6.0	9.7	48.3	9.7	16.9
< 6.0	12.1	34.5	16.5	4.4
		61.7		
		37.0		
		93.7		
Mean < 5.1	12.8	49.2	16.8	10.3
S.E.	1.8	8.3	2.6	3.1

Table 4: Blood bradykinin level in intraperitoneally challenged guinea-pig. Not more than one sample was collected from one animal.

Figure 4 shows that after anaphylactic shock the blood bradykinin level rose in 30 minutes; samples withdrawn after one hour were found to be the most active. The activity appeared to decline by $1\frac{1}{2}$ hours, and was down almost to control levels by 2 hours.

Intravenous challenge:

In view of results obtained from intraperitoneally shocked animals, some guinea-pigs were challenged intravenously. In order to keep them alive and permit withdrawal of samples, mepyramine maleate 5 mg/kg was

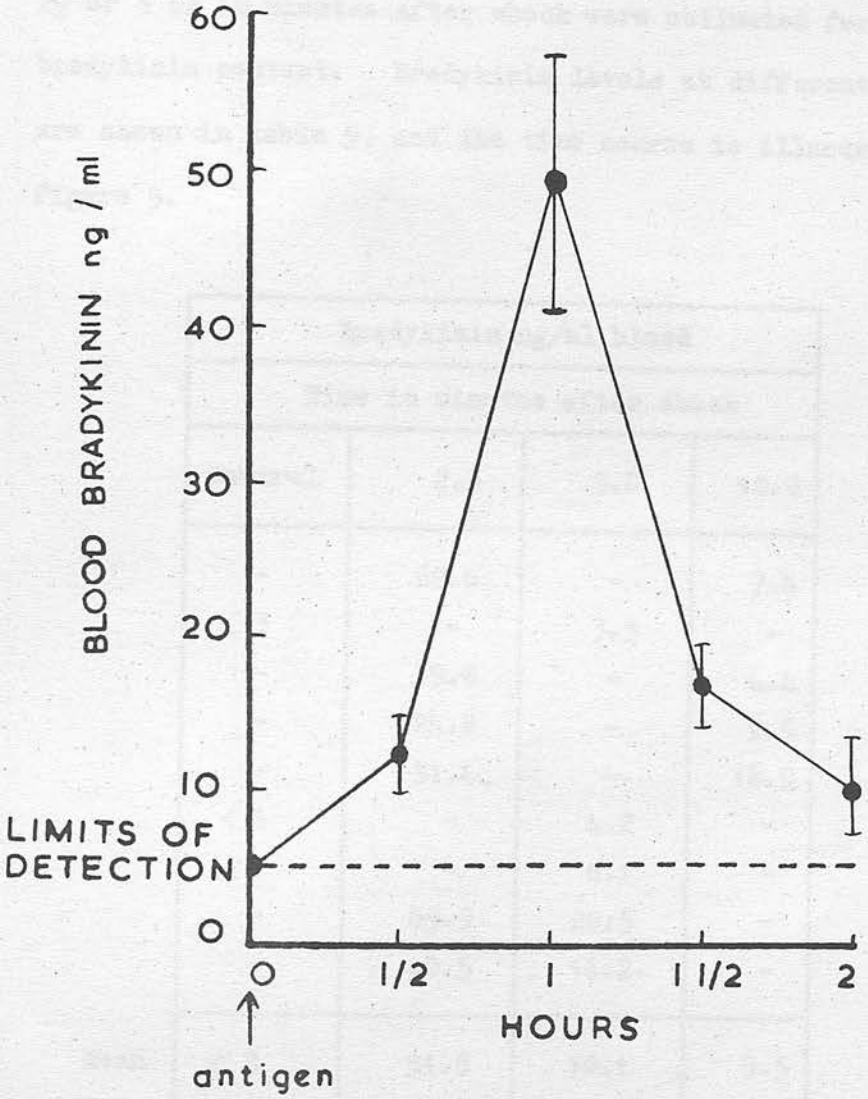


Fig. 4: Blood bradykinin level in guinea-pig after intraperitoneal challenge.
Vertical bars represent standard error.

injected intraperitoneally 45 minutes before they were challenged intravenously with 0.2 ml 1% solution of crystalline egg albumin. Samples of blood taken before or $2\frac{1}{2}$ or 5 or 10 minutes after shock were estimated for bradykinin content. Bradykinin levels at different times are shown in table 5, and the time course is illustrated in figure 5.

	Bradykinin ng/ml blood			
	Time in minutes after shock			
	Control	2.5	5.0	10.0
	-	69.6	-	7.4
	< 3	-	3.5	-
	-	39.0	-	4.4
	-	24.2	-	9.5
	-	51.4	-	16.9
	< 3	-	4.2	-
	< 3	-	6.1	-
	-	85.9	20.5	-
	-	40.5	16.2	-
Mean	< 3	51.8	10.1	9.5
S.E.		9.2	3.5	2.7

Table 5: Blood bradykinin levels in mepyramine-protected guinea-pigs challenged with soluble antigen given intravenously; each horizontal row represents one animal.

Each horizontal row in table 5 represents the same animal. Not more than two samples were taken from any one animal, so as to avoid any possible alteration of blood bradykinin

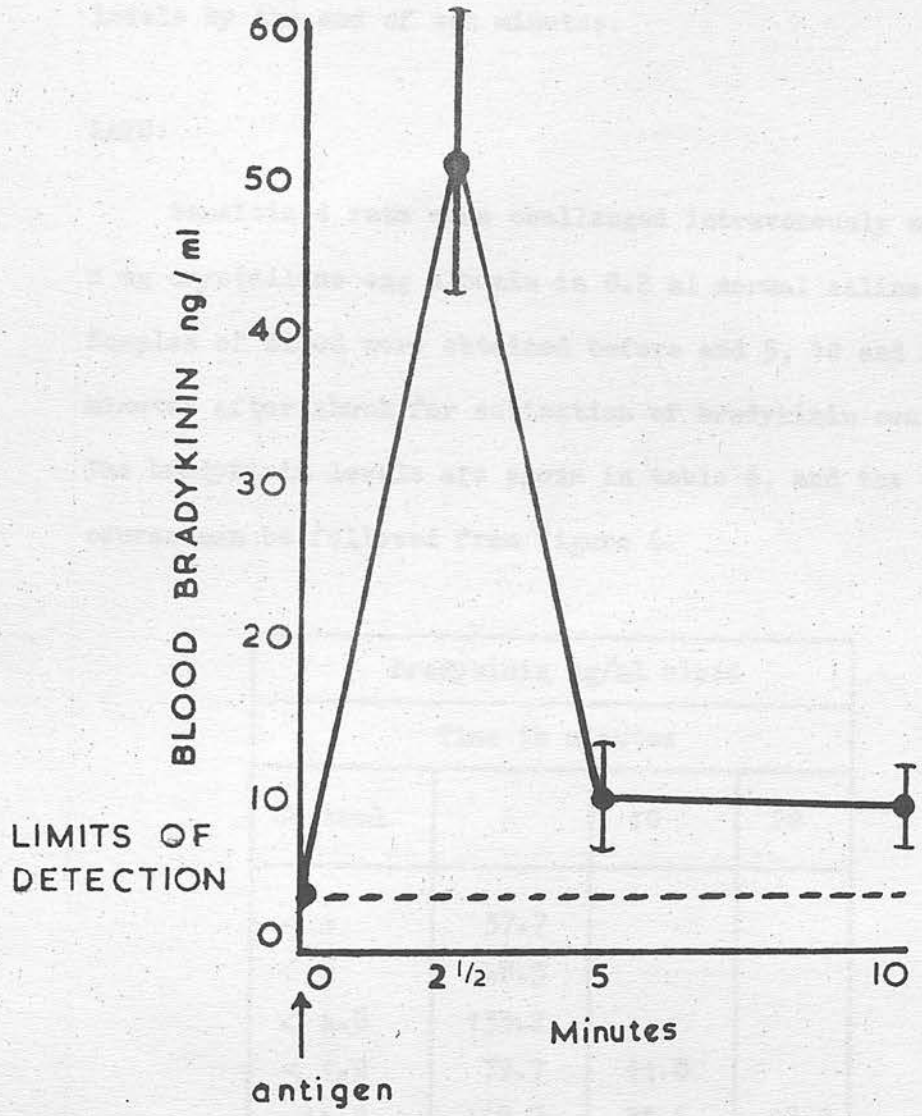


Fig. 5: Blood bradykinin level in guinea-pig after intravenous challenge; vertical bars represent standard error

level in the later samples. It appeared that the samples were most active $2\frac{1}{2}$ minutes after shock, but activity decreased rapidly, and had almost returned to control levels by the end of ten minutes.

RATS:

Sensitised rats were challenged intravenously with 2 mg crystalline egg albumin in 0.2 ml normal saline. Samples of blood were obtained before and 5, 10 and 20 minutes after shock for estimation of bradykinin content. The bradykinin levels are shown in table 6, and the time course can be followed from figure 6.

	Bradykinin ng/ml blood			
	Time in minutes			
	Control	5	10	20
	< 6	37.7		
	< 6	48.3		
	< 4.8	133.2		
	< 1.2	79.7	11.0	
	14.5	108.7	73.6	28.0
	5.7		7.3	
	< 2.5	21.2	5.7	
	< 0.5		7.2	
Mean	< 5.1	71.5	21	
S.E.		17.7	13.2	

Table 6: Blood bradykinin level in intravenously challenged rats.

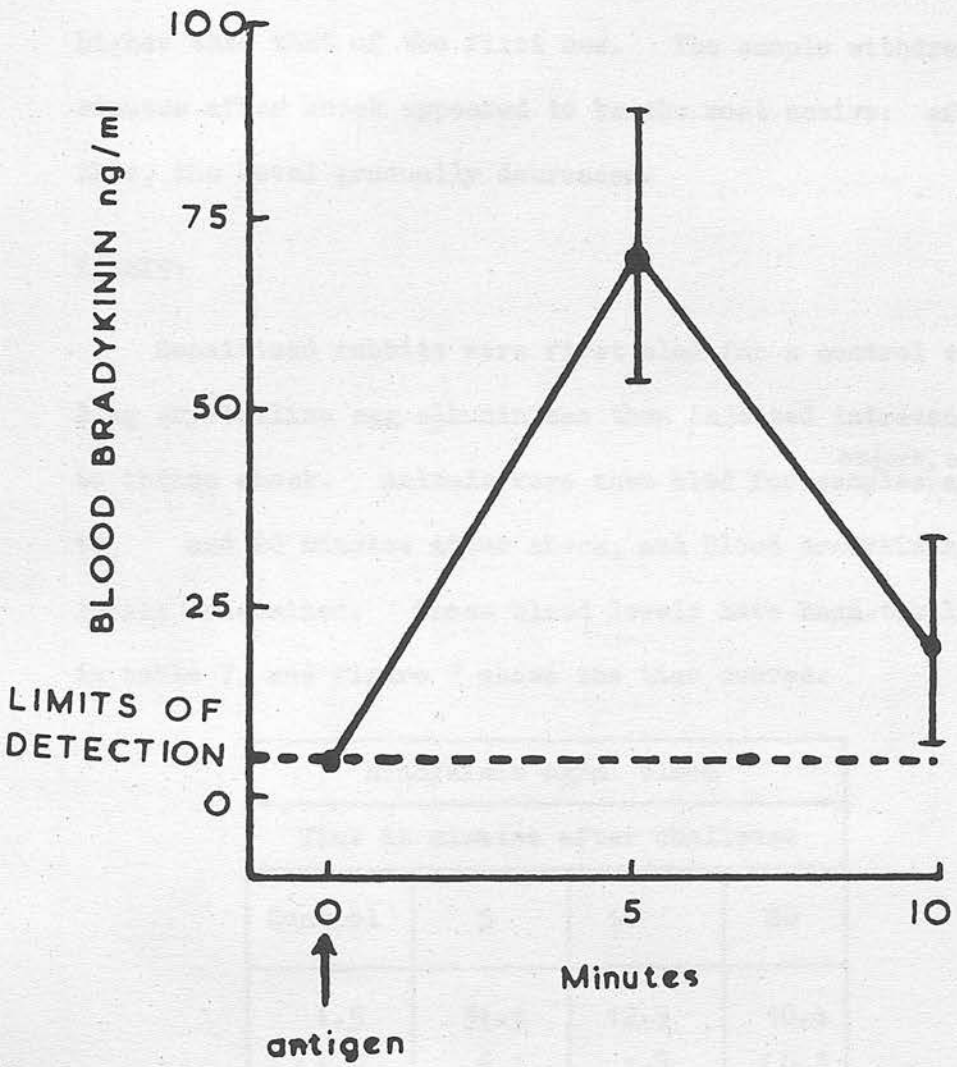


Fig. 6: Blood bradykinin level in rat after intravenous challenge.
Vertical bars represent standard error.

Each horizontal row represents one animal. Most animals were bled only twice. It was found that when two control samples of blood (1-2 ml each) were withdrawn from an unsensitised animal, at five minutes interval, the bradykinin content of the later sample was not appreciably higher than that of the first one. The sample withdrawn 5 minutes after shock appeared to be the most active: after this, the level gradually decreased.

RABBIT:

Sensitised rabbits were first bled for a control sample; 2 mg crystalline egg albumin was then injected intravenously to induce shock. Animals were then bled for samples before, and at 5, 10 and 20 minutes after shock, and blood bradykinin levels determined. These blood levels have been tabulated in table 7, and figure 7 shows the time course.

	Bradykinin ng/ml blood			
	Time in minutes after challenge			
	Control	5	10	20
	1.5	31.1	12.9	10.4
	<1.0	6.2	1.8	<0.3
	<1.4	6.7	6.4	4.9
	<1.5	9.2	4.0	<2.2
Mean	<1.3	13.3	6.3	4.4
S.E.		5.9	2.4	1.9

Table 7: Blood bradykinin levels in intravenously challenged rabbits

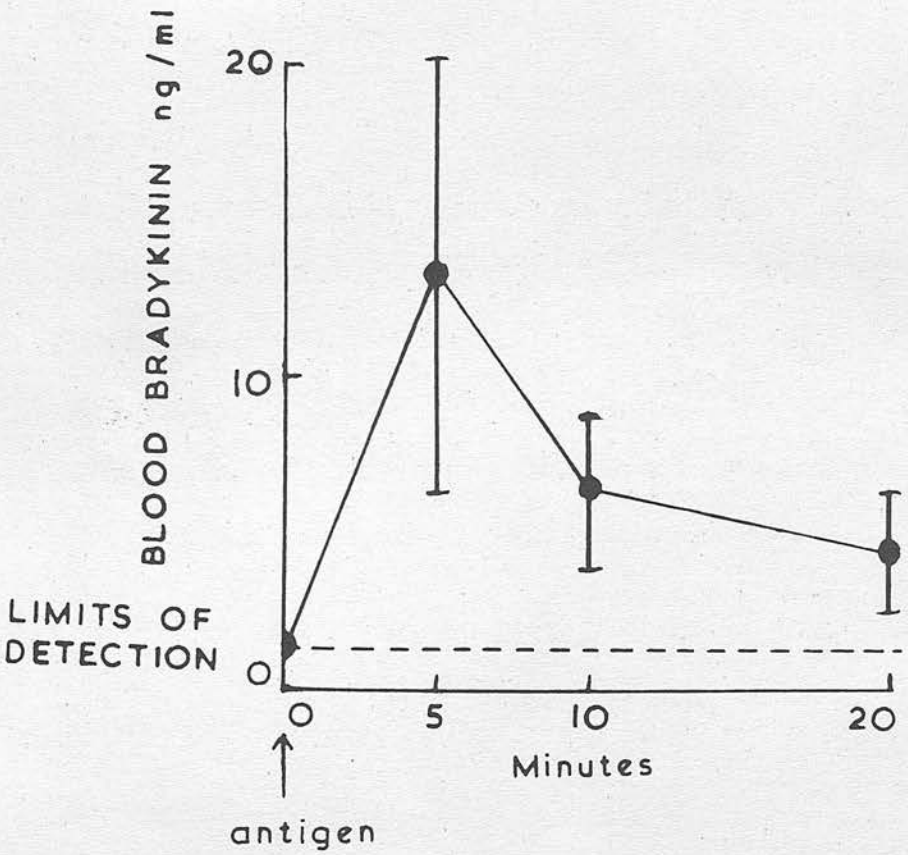


Fig. 7: Blood bradykinin level in rabbit after intravenous challenge.
Vertical bars represent standard error.

As in the rat, the 5 minute sample appeared to be the most active, after which activity gradually decreased. In general, the level of peak activity in rabbit was less than in guinea-pig and rat.

Further efforts were made to identify the active principle present in the blood extracts as bradykinin.

Chymotrypsin digestion:

Active samples of extracted guinea-pig, rat and rabbit blood collected during anaphylactic shock were incubated with salt-free chymotrypsin (0.5 mg/ml blood) for 30 minutes at 37°C. The reaction mixture was then boiled for a few minutes and cooled. The chymotrypsin, so treated, and present in amounts in which it occurred in the reaction mixture did not have any oxytocic activity on the isolated rat uterus preparation. In all three species it was found that chymotrypsin digestion destroyed more than 80% of the activity in the extracted sample. This is illustrated in tracings shown in figures 8, 9 and 10.

Parallel quantitative assay:

Extracts of active samples of blood drawn from all three species were assayed against bradykinin on rat uterus, guinea-pig ileum and rat duodenum. These are shown in table 8. The values obtained with different test preparations are in reasonable agreement. The highest index of discrimination, as described by Gaddum (1955) was 2.0. It was important to employ rat

duodenum from a qualitative point of view since bradykinin relaxes rat duodenum, while almost all other smooth muscle stimulant substances contract it.

Blood extract from	Bradykinin ng/ml blood as estimated on			Highest index of discrimination
	Rat uterus	Guinea-pig ileum	Rat duodenum	
Guinea-pig	52.8	108.0	60.0	2.0
Rat	30.4	55.8	56.0	1.8
Rabbit	31.1	27.9	17.0	1.8

Table 8: Parallel quantitative estimation of active samples compared with synthetic bradykinin on different test objects.

The effect of active guinea-pig blood extract on the blood pressure of normal rat was noted. The rat was made resistant to the hypotensive effect of histamine and 5-hydroxytryptamine with mepyramine (20 mg/kg) and 2-bromo-lysergic acid (2.5 mg/kg). Intravenous administration of the extract produced fall in blood pressure. However, as much as 1.5 ml extract had to be administered to produce this effect, and so further quantitative examination was not done. The large amount of extract needed for lowering blood pressure was not surprising in view of the fact that about 100 ng bradykinin was needed to produce a hypotensive response in this animal.

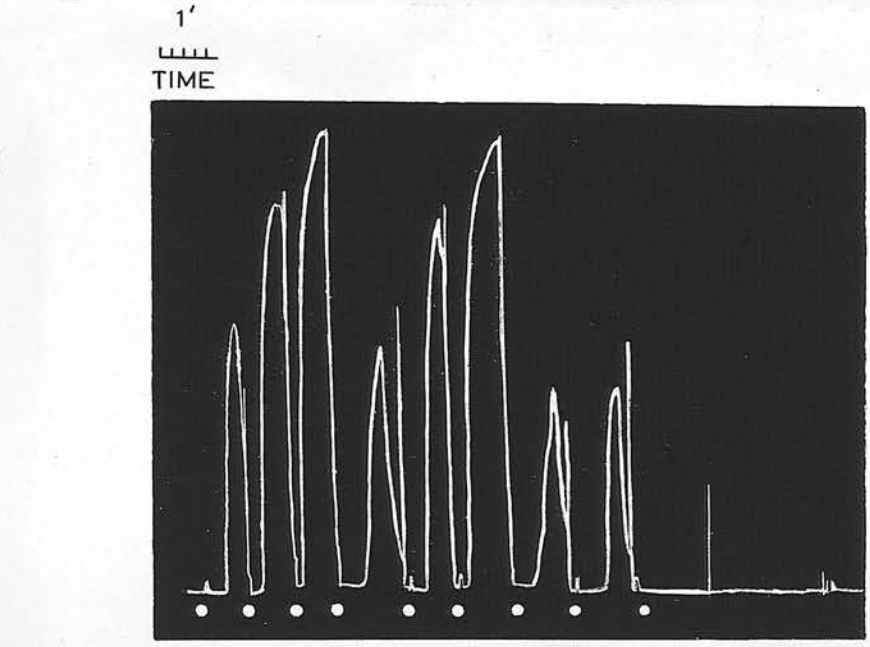


Fig. 8: Rat uterus: Loss of activity on chymotrypsin-digestion (=UC) of extract from guinea-pig blood (=U) collected one hour after intra-peritoneal challenge. BK = bradykinin.

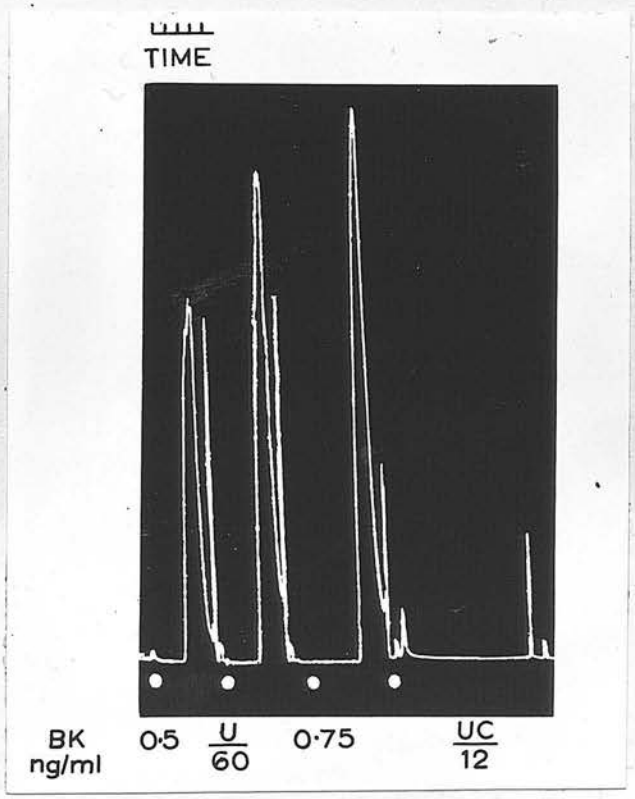


Fig. 9: Rat uterus: Loss of activity on chymotrypsin-digestion (= UC) of extract from rat blood (=U) collected five minutes after intravenous challenge. BK = bradykinin.

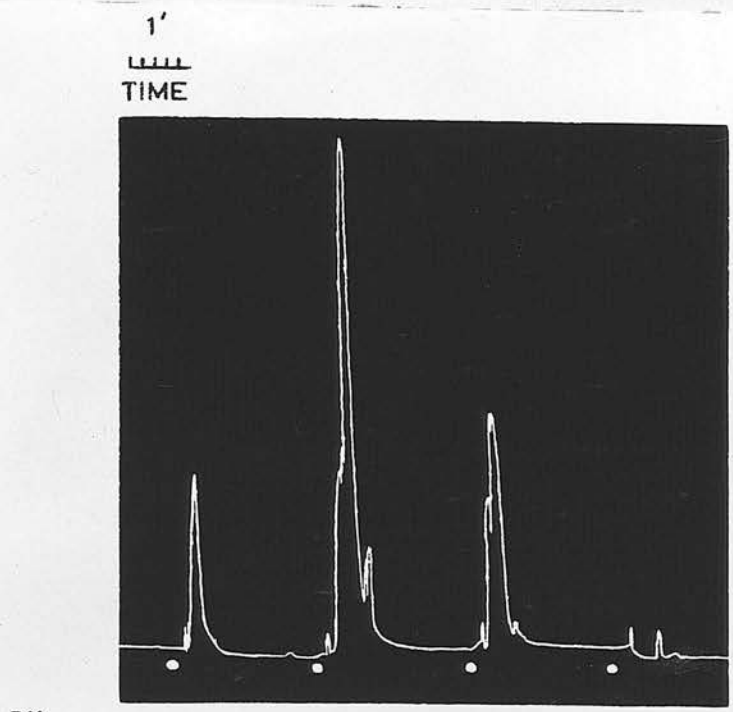


Fig. 10: Rat uterus: Loss of activity on chymotrypsin digestion (=UC) of extract from rabbit blood (=U) collected five minutes after intravenous challenge. BK = bradykinin.

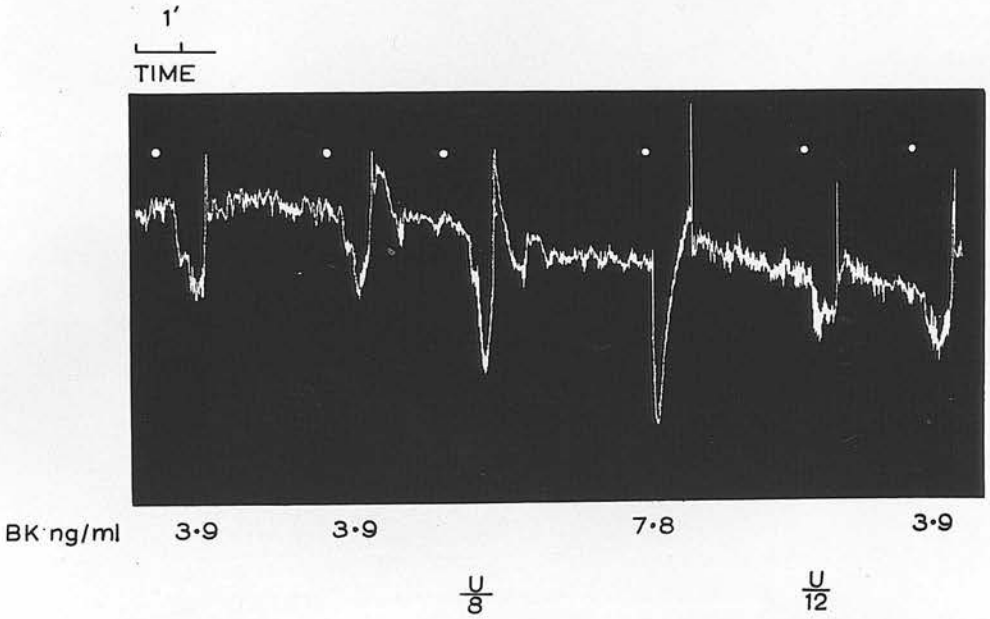
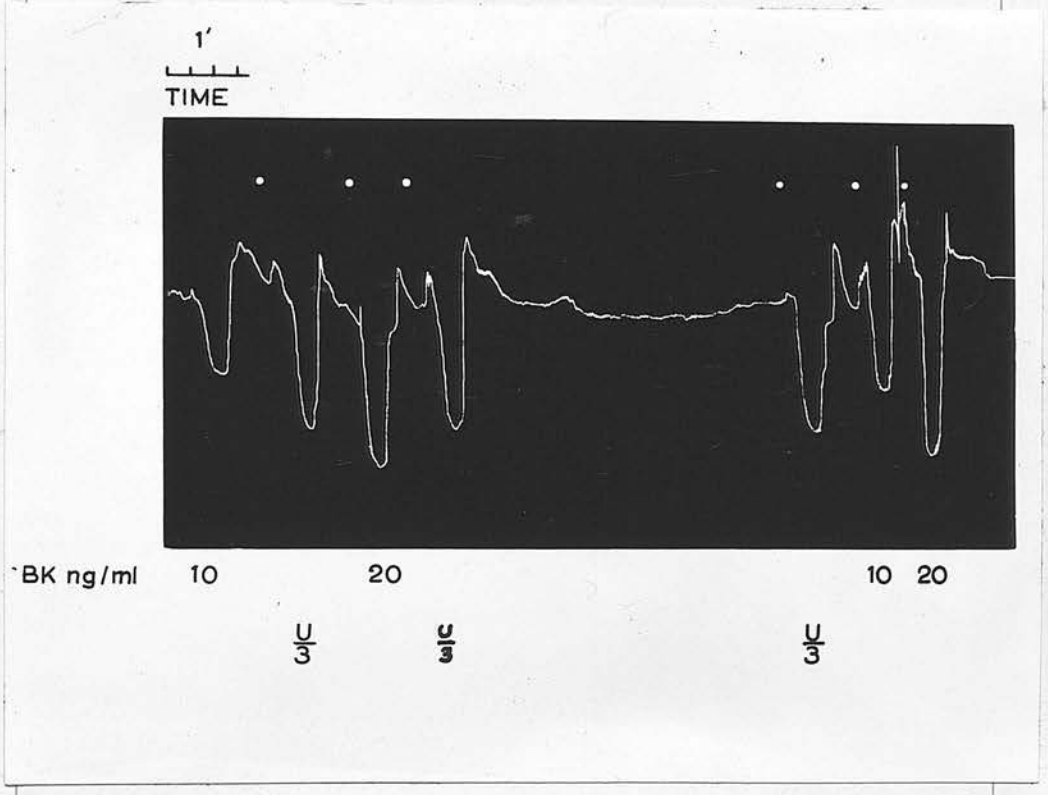


Fig. 11(a): Relaxation of rat duodenum with extract (=U) of post-shock blood from guinea-pig. BK = bradykinin.

(b)



(c)

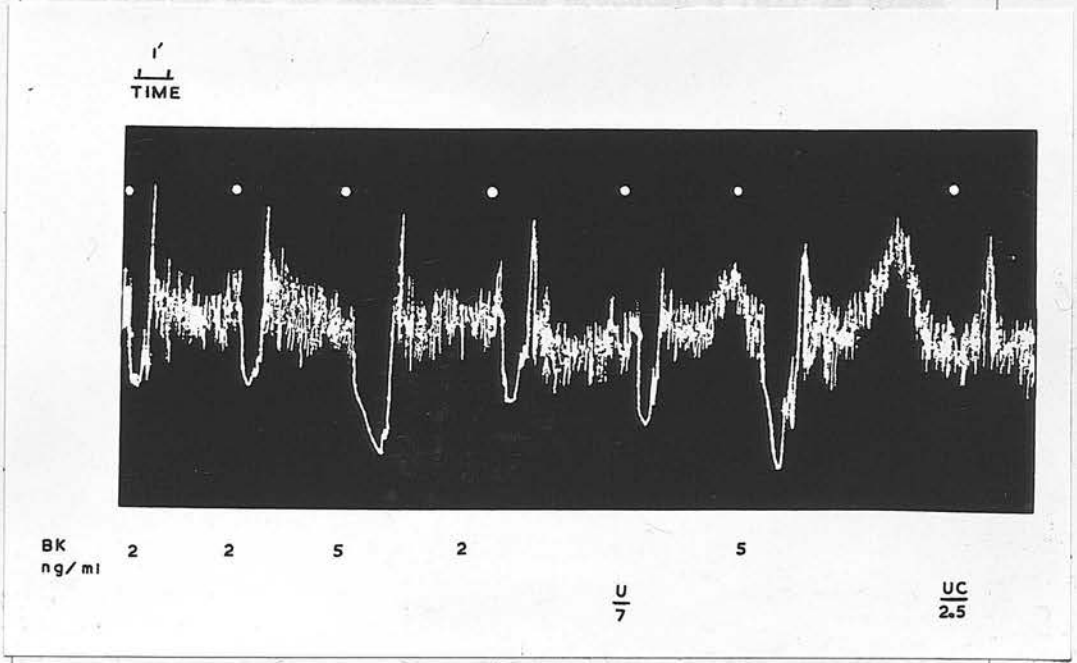


Fig. 11(b) and (c): Relaxation of rat duodenum with extract (=U) of post-shock blood: (b) from rat, extract dissolved in 1.25 vols de Jalon solution; (c) from rabbit, UC = chymotrypsin-digested rabbit blood extract.
BK = bradykinin.

RAT BLOOD PRESSURE EXPERIMENTS:

Sensitised rats were prepared for blood pressure recording and mepyramine maleate (15 to 25 mg/kg) and 2-bromo-lysergic acid (2 to 2.5 mg/kg) were given until the hypotensive responses to 2 or 3 times the previously effective dose of histamine and 5-hydroxytryptamine was completely suppressed. It was necessary to administer these drugs, particularly 2-bromo-lysergic acid in divided doses, as otherwise a severe fall of blood pressure followed by death occurred. Even with divided doses, there was slight lowering in the blood pressure level. Bradykinin produced a temporary fall of blood pressure in these animals. Intravenous injections of 2 mg crystalline egg albumin in 0.2 ml normal saline produced a fall in blood pressure, which was prolonged in almost all instances, and sometimes resulted in death: in one instance however (figure 12) it returned to normal in a comparatively short period. Blood pressure records from experiments showing the more usual type of response are shown in figure 13.

To summarise, experiments in this section showed that the bradykinin level in blood rose following anaphylaxis in guinea-pig, rabbit and rat, and the time-course in these species was studied. The identity of the active material was confirmed by chymotrypsin digestion and parallel quantitative assay. Indirect evidence from rat blood pressure experiments supported the conclusion that bradykinin contributes materially to the pattern of anaphylaxis.

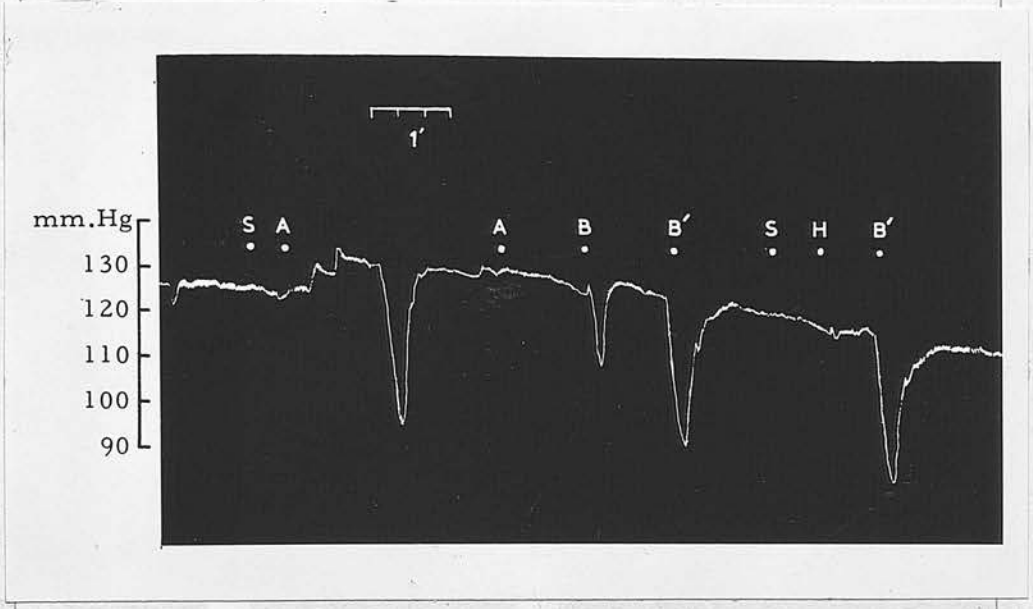


Fig. 12

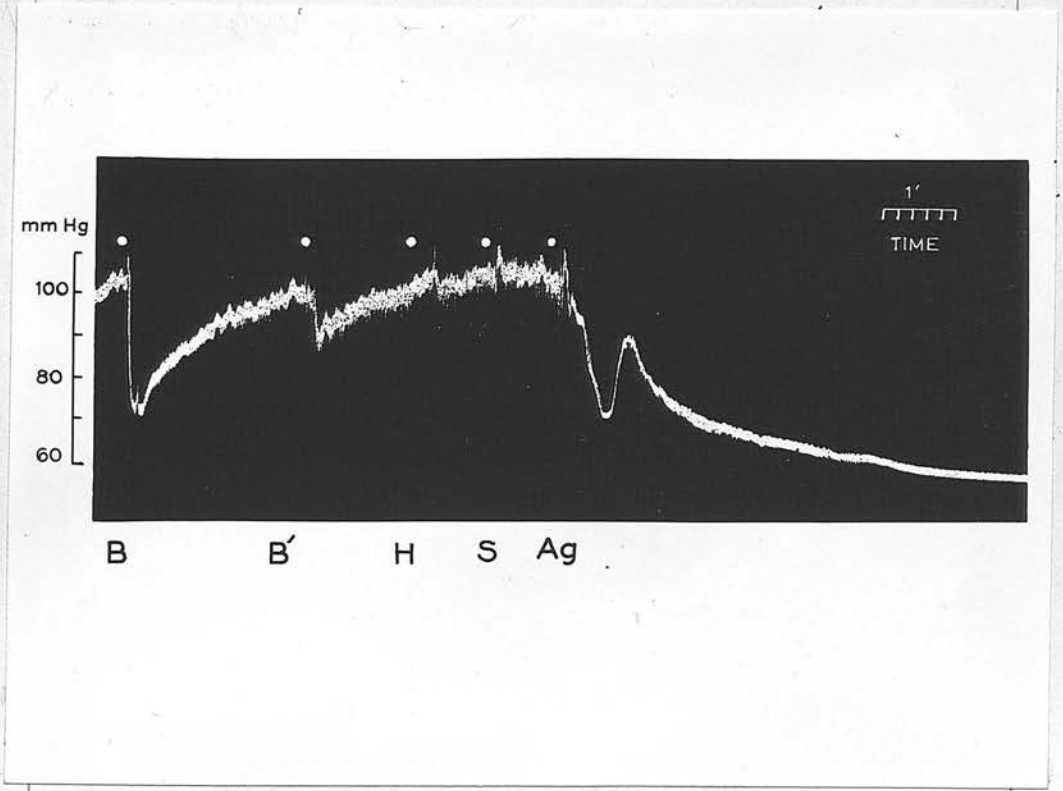


Fig. 13(a)

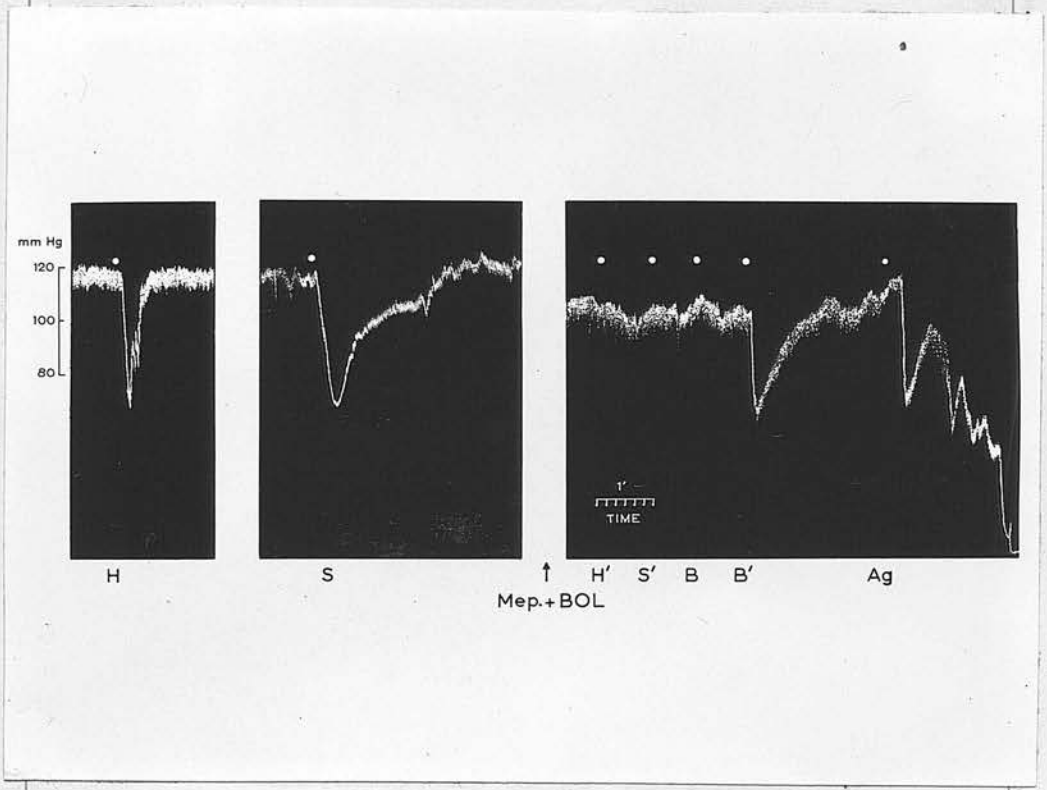


Fig. 13(b): Anaphylactic hypotension in rat after the effect of histamine and 5-hydroxytryptamine has been excluded. Ag = crystalline egg albumin antigen 2 mg; H, H′ = histamine base 20 and 60 μg/kg respectively; S, S′ = 5-hydroxytryptamine base 4 and 10 μg/kg respectively; Mep. + BOL = mepyramine maleate 20 mg/kg and 2 bromo-lysergic acid 2.3 mg/kg respectively in divided doses over an interval of 2 hours; B, B′ = bradykinin 0.6 and 1.2 μg/kg respectively.

SECTION III

EFFECT OF ANAPHYLACTIC SHOCK ON BRADYKININOGEN LEVEL IN PLASMA

EFFECT OF ANAPHYLACTIC SHOCK ON BRADYKININOGEN LEVEL IN PLASMA:

Raised blood bradykinin levels during anaphylaxis in vivo suggested a concurrent depletion of blood bradykininogen content. The experiments in this section were designed to find out a suitable method for the evaluation of bradykininogen in blood, and to detect possible change. It is not possible to express bradykininogen content of plasma in terms of its weight as the material has never been obtained pure. Furthermore, the basis of quantitation is biological assay of the bradykinin which can be obtained from the precursor. Therefore the amounts of bradykinin obtained by trypsin digestion from 1 ml plasma have been accepted as the meaningful indication of bradykininogen levels.

Development of a suitable bradykininogen assay method for small samples of plasma:

A method for estimation of the bradykininogen content of small volumes of blood had to be evolved before changes due to anaphylaxis could be studied. Two procedures were available: (1) The use of fresh plasma as substrate with trypsin: A few experiments showed that yields were very low with this method, less than 0.25 µg bradykinin per ml plasma. The low yield was surmised to be due to interfering enzymic activity resulting in spontaneous formation and destruction of bradykinin. (2) Initial

Fig. 12: Anaphylactic hypotension in rat after the exclusion of histamine and 5-hydroxytryptamine. The rat had been given 15 mg/kg mepyramine maleate and 2 mg/kg 2-bromo-lysergic acid. H = histamine base 67 µg/kg; S = 5-hydroxytryptamine base 2 µg/kg; B, B′ = bradykinin 0.09 and 0.18 µg/kg respectively; A = 2 mg crystalline egg albumin antigen.

Fig. 13(a): Anaphylactic hypotension in rat after the effect of histamine and 5-hydroxytryptamine has been excluded by previous administration of mepyramine maleate (25 mg/kg) and 2-bromo-lysergic acid (2.5 mg/kg); H = histamine base 50 µg/kg; S = 5-hydroxytryptamine base 5 µg/kg; B, B′ = bradykinin 1.0 and 0.6 µg/kg respectively; Ag = crystalline egg albumin antigen 2 mg.

(Facing p. 81)

denaturation with ammonium sulphate, as described by Holdstock, Mathias and Schachter (1957), to prevent such enzymic interference. This method could not be adopted because comparatively large amounts were needed, and the experimental manipulations including dialysis were likely to lead to bradykininogen loss.

A simple procedure suitable for small samples, and not involving dialysis was sought. The method described below, based on denaturation with ethanol was found suitable.

Arterial blood was collected from sensitised guinea-pigs via a polythene cannula into siliconed glass centrifuge tubes containing heparin (1 I.U./ml). Plasma was separated by centrifugation and added to 4 volumes of ethanol at 2°C being vigorously mixed to give a fine precipitate. The mixture was heated at 80-90°C for 20 minutes and centrifuged at about 1,300 g and the supernatant drained off and discarded; the precipitate was washed in distilled water. The precipitate was then suspended in 2 ml 0.1 M phosphate buffer at pH 7.4, and incubated with trypsin (≡ 0.5 mg/ml plasma) at 37°C for 20 minutes. After 20 minutes, 2 volumes of hot ethanol was added to the reaction mixture, and the vessel was kept in a boiling water bath for a few minutes. It was then cooled and centrifuged for 20 minutes approximately at 1,300 g. The supernatant was dried in partial vacuum at 35°-40°C in a slow stream of nitrogen. The dried material was dissolved in de Jalon solution and assayed on rat uterus for bradykinin content.

Changes in plasma bradykininogen level before and after anaphylaxis:

After collection of a 1-2 ml control sample of blood from sensitised guinea-pigs, 2 mg aluminium-hydroxide-adsorbed antigen in 3 ml saline was injected intraperitoneally. Three hours later, another sample of blood was taken. Bradykininogen content in each sample was estimated according to the procedure described above. Since the rise in blood bradykinin level practically disappears after two hours, it was felt that by three hours, bradykinin formation would have ceased. In three animals, in addition to samples for bradykininogen estimation, a sample of blood was obtained one hour after challenge so as to relate the peak blood bradykinin level to the bradykininogen depletion in those animals. The results are shown in table 9 and figure 14;

	Bradykininogen ≡ bradykinin µg/ml plasma				Blood bradykinin ng/ml
	Control	Post-shock	Absolute loss	% loss	
	8.4	4.17	4.23	50.3	
	8.79	4.45	4.34	49.4	
	5.2	4.97	0.23	4.6	
	6.9	3.35	3.55	51.4	
	8.86	5.87	2.99	33.7	37.0
	13.89	7.2	6.69	48.1	61.7
	18.75	10.12	8.63	46.0	93.7
	19.1	8.9	10.2	53.4	
	23.0	10.1	12.9	56.1	
	15.72	11.21	4.51	28.7	
Mean	12.86	7.03	5.83	42.2	
S.E.	1.92	0.91	1.12	5.0	

Table 9: Bradykininogen content in guinea-pig plasma before and after anaphylaxis.

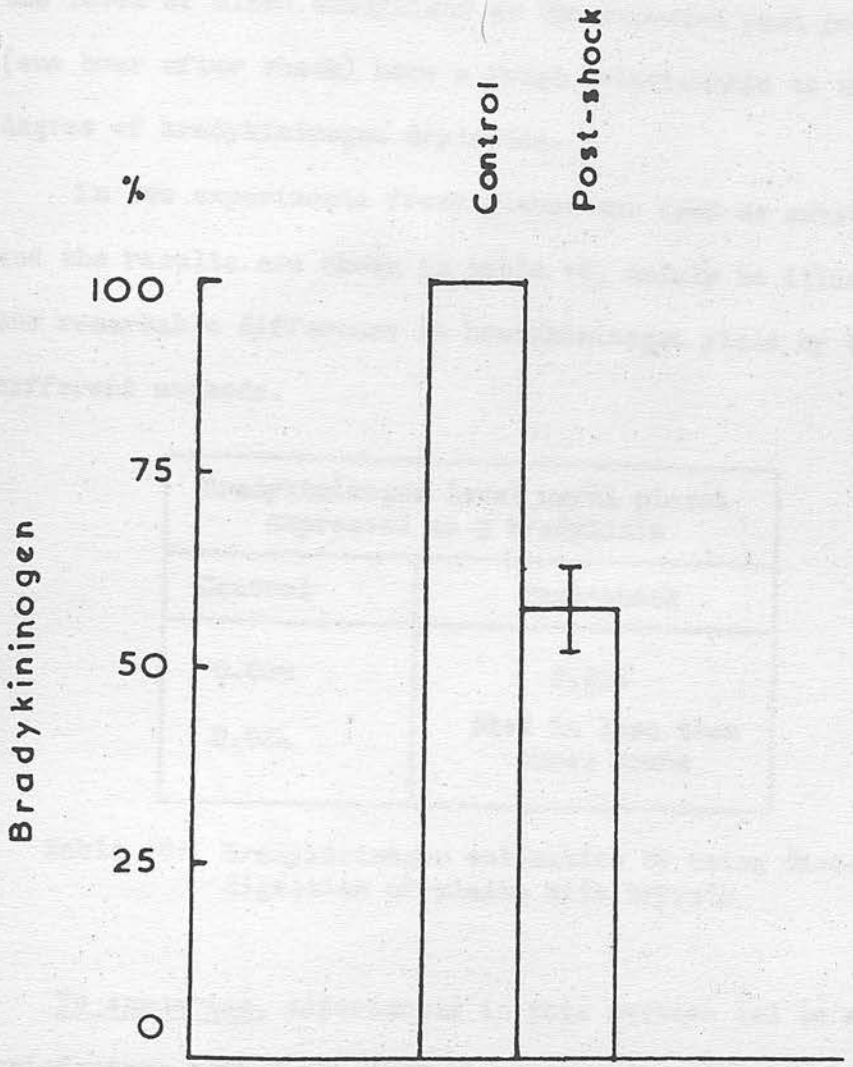


Fig. 14: Depletion of plasma bradykininogen after retarded shock. The vertical bar represents standard error

it will be seen that in three hours after shock, the blood was considerably depleted of bradykininogen. Furthermore, the level of blood bradykinin at the expected peak period (one hour after shock) bore a rough relationship to the degree of bradykininogen depletion.

In two experiments fresh plasma was used as substrate, and the results are shown in table 10, mainly to illustrate the remarkable difference in bradykininogen yield by the different methods.

Bradykininogen level µg/ml plasma expressed as ≡ bradykinin	
Control	Post-shock
0.069 0.024	0.026 Died in less than three hours

Table 10: Bradykininogen estimation by using direct digestion of plasma with trypsin

To summarise, experiments in this section led to a satisfactory method for bradykininogen assay in small samples; employing this method, anaphylactic shock was shown to deplete blood bradykininogen level considerably.

SECTION IV

FURTHER EXPERIMENTS ON BRADYKININ FORMATION:

(1) ANTIGEN-ANTIBODY REACTION IN BLOOD *IN VITRO*, and

(2) ANTIGEN-ANTIBODY REACTION IN PERITONEAL FLUID *IN VIVO*.

FURTHER EXPERIMENTS ON BRADKININ FORMATION: (1) ANTIGEN-ANTIBODY REACTION IN BLOOD IN VITRO, AND (2) ANTIGEN-ANTIBODY REACTION IN PERITONEAL FLUID IN VIVO

The experiments described in Section II showed a raised level of bradykinin in the circulating blood during anaphylaxis *in vivo*. The experiments in this section were designed to show if blood contained all the factors necessary for the production of bradykinin during anaphylaxis, and if bradykinin was detectable elsewhere.

Blood was collected from sensitised guinea-pigs and rats via a polythene arterial cannula in polythene containers containing heparin (final concentration in mixture 1 I.U./ml blood). Blood was handled throughout with polythene or siliconed glassware. 2 ml aliquots of blood were incubated with 0.2 ml normal saline as control or with 2 mg crystalline egg albumin in 0.2 ml saline. Guinea-pig blood was incubated for two minutes and rat blood for five minutes. After incubation, control and experimental samples were immediately mixed with ethanol, as described in section I and estimated for bradykinin content. The estimated levels are given in tables 11 and 12 which show that the challenged and control samples of blood contained virtually the same insignificant amount of bradykinin.

Guinea-pig blood: Incubation period two minutes	
Before shock ng/ml	After shock ng/ml
<3	<3
<8	<8

Table 11: Bradykinin level in sensitised guinea-pig blood challenged in vitro

Rat blood: Incubation period five minutes	
Before shock ng/ml	After shock ng/ml
5.1	8.2
4.1	5.1
5.6	5.2

Table 12: Bradykinin level in sensitised rat blood challenged in vitro

Antigen-antibody reaction in the peritoneal cavity of rat according to the method of Rapp (1962) followed by biological assay of the peritoneal fluid:

Male rats (100 to 200 g bodyweight) were injected intraperitoneally with 0.6 µg anti-BSA antibody in 0.2 ml saline or Tyrode solution. Four hours later, 2 mg antigen in 5-7 ml of normal saline or Tyrode was injected intraperitoneally. The rat was killed ten minutes later, and the peritoneal fluid recovered by means of a polythene pipette. For control purposes, some animals were injected with antibody only, but no antigen; some others were injected with antigen only, but no antibody. The

recovered peritoneal fluid was kept on ice until estimated for bradykinin-like activity on rat uterus the same day. The estimated bradykinin content in these samples is shown in table 13. It is to be noticed that while antigen-antibody reaction was followed by bradykinin-like activity in the peritoneal fluid, similar activity was found in control samples also.

Physiol. solution employed	Antibody	Antigen	Bradykinin-like activity, in ng/ml fluid
Saline	+	+	97.6
Saline	+	+	48.0
Saline	+	-	65.1
Saline	-	+	31.6
Tyrode	+	-	14.2
Tyrode	-	+	18.3
Tyrode	+	+	16.8
Tyrode	+	+	45.0

Table 13: Bradykinin-like activity in peritoneal fluid in rats following antigen-antibody reaction.

To summarise, experiments described in this section showed practically no change in the bradykinin content of sensitised blood challenged in vitro; and no significant increase in bradykinin-like activity in rat peritoneal fluid after anaphylaxis.

SECTION V

BRADYKININ-FORMING ACTIVITY DURING ANAPHYLAXIS IN VITRO: SENSITISED GUINEA-PIG SKIN

BRADYKININ-FORMING ACTIVITY IN GUINEA-PIG SKIN PERFUSATE AFTER ANAPHYLAXIS

These experiments were done to ascertain whether bradykinin might be formed in skin during anaphylaxis *in vitro*.

Development of a suitable skin perfusion method:

The existing method of Feldberg and Paton (1951) involved thermocautery and thus probably activates proteolytic enzymes of the skin. It was therefore considered unsuitable, and a method was sought which would avoid activation of proteolytic enzymes as far as possible.

The procedure described below was found to be satisfactory. This was based on arterial perfusion of an isolated piece of skin from the hip, suspended in air in a warm chamber. The effluent flows out of the cut edges of the skin, and is collected.

Sensitised guinea-pigs of 400 to 500 g bodyweight were used. The lower half of the body was shaved with electric clippers. Under urethane anaesthesia, a vertical incision was made from the inguinal ligament downwards along the centre of the anterior aspect of the thigh. The femoral artery and its profunda femoris branch were carefully dissected. All branches of the femoral artery except the superficial circumflex iliac artery were

divided between ligatures. Particular care was taken not to miss any cutaneous or muscular branches. The femoral artery was divided between ligatures at a point approximately 5 to 10 mm distal to the origin of the profunda femoris artery. Through a nick in the profunda femoris artery, a polythene cannula was pushed upwards and tied so that its tip was just distal to the origin of the superficial circumflex iliac artery(see figure 15). The femoral artery was divided between ligatures just above the origin of the superficial circumflex iliac artery. The area of skin supplied by the superficial circumflex femoral artery was excised along with its vascular supply. It comprises of an area of 7 to 12 cm^2 extending over the posterolateral aspect of the upper quarter of the thigh. The whole preparation was suspended by a thread in a chamber at 37°C. The isolated piece of skin was perfused with Tyrode at 37°C under a pressure head of 150 cm. The effluent from the cut edges of the skin was collected only after the perfusate appeared to be free of blood. The entire perfusion assembly is represented diagrammatically in figure 16. Blood-free and adequately perfused preparations could be obtained by this method, as tested with India ink injection following perfusion (see figure 17). (* vide p. 100, at the end of this section, for an alternative skin area suitable for this preparation).

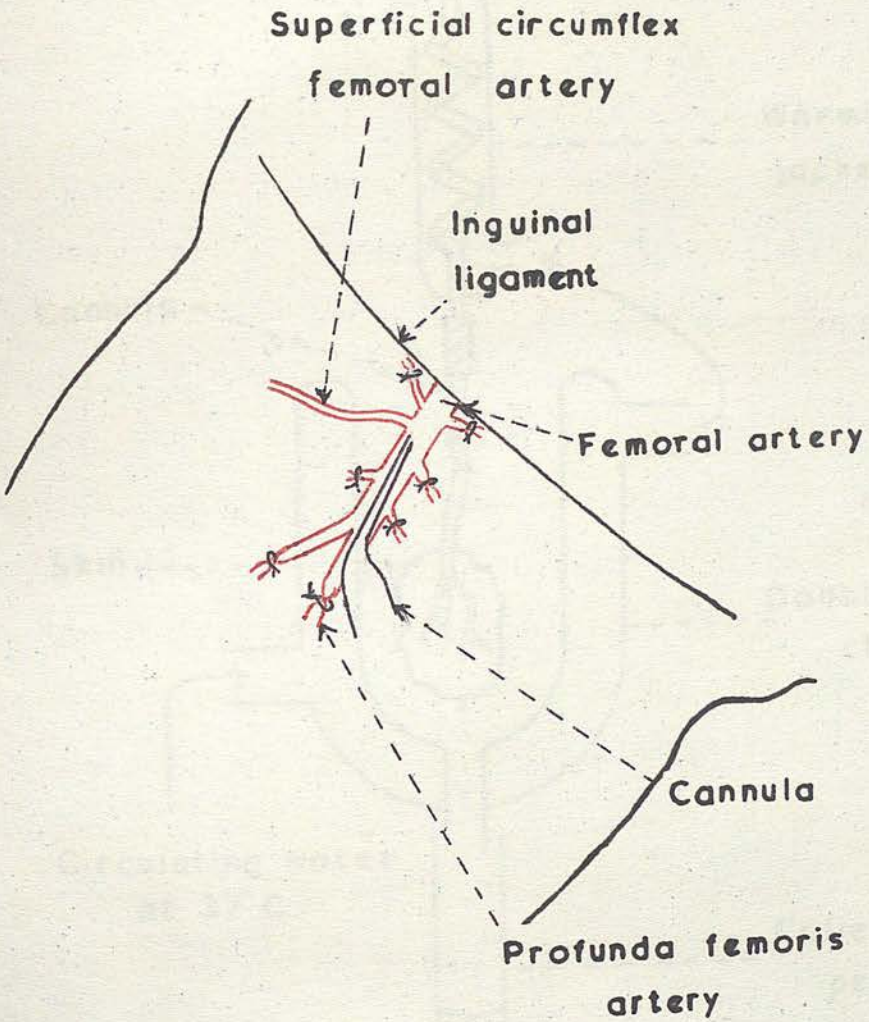


Fig. 15: Dissection for isolated skin preparation

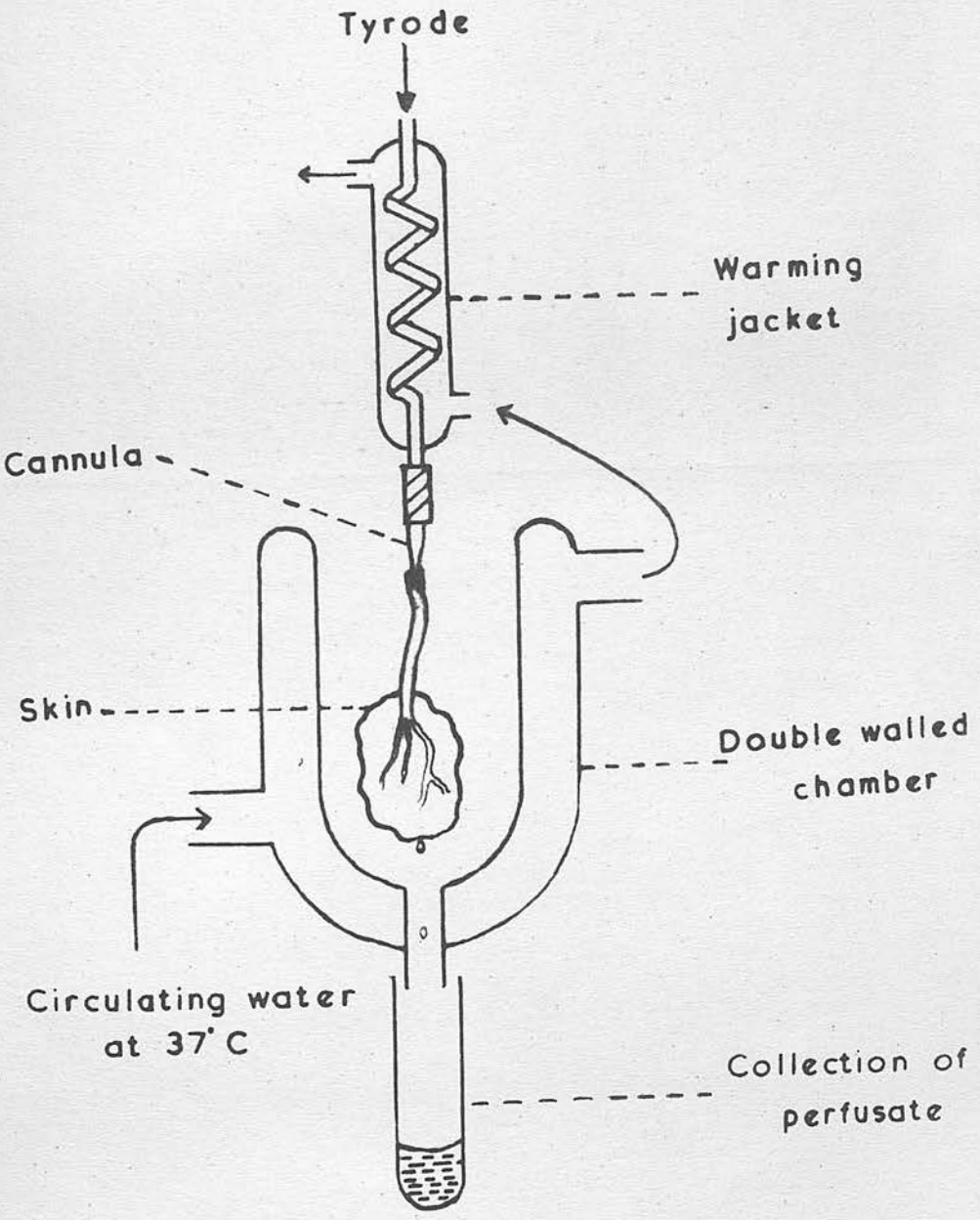


Fig. 16: Assembly for guinea-pig skin perfusion

(a) (b) (c)

Figure 17: Isolated skin preparation: (a) unperfused; (b) perfused from the other limb of the same animal; (c) India ink injected after perfusion.

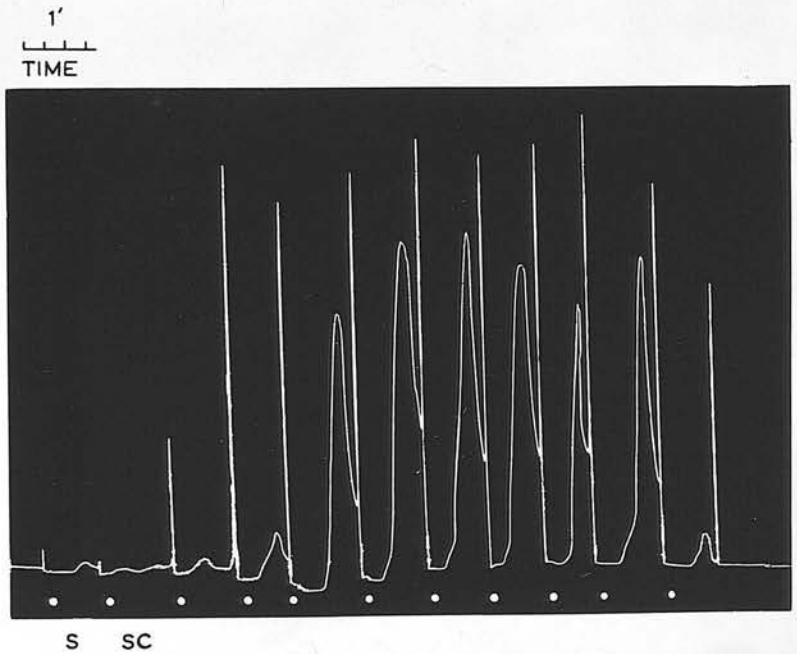
Experimental:

Effluent was collected over one-minute periods before and at various periods after anaphylactic shock. Anaphylaxis in vitro was induced by injecting 2 mg crystalline egg albumin in 0.2 ml Tyrode or saline at 37°C into the perfusing solution just before it reaches the preparation. Neither control nor post-shock samples showed any bradykinin-like activity, when tested on rat uterus. Some samples of perfusate collected in the first minute after shock, showed some oxytocic activity in concentrations as high as twofold dilution. This would represent about 1 ng bradykinin in the entire sample and can be ignored. To test for bradykinin-forming

Fig. 18: Optimum period of incubation of post-shock skin perfusate with pseudoglobulin substrate: Rat uterus, auxotonic lever; perfusate collected over 75 seconds after shock and made up to 4.5 ml with Tyrode and incubated with 0.5 ml substrate. Dilution of aliquots (= x 20) drawn at different times shown in illustration. SC = control perfusate + substrate incubated for 10 mins and S = substrate, both diluted x 20. BK = bradykinin.

(Facing p. 96)

activity, the perfusate was incubated at 37°C with heated dog plasma pseudoglobulin solution equivalent to 0.5 ml plasma. Preliminary experiments were carried out to investigate the effect of varying incubation periods. It was found that the optimum incubation period for bradykinin-formation was about 10 minutes; activity gradually declined after this period, until at 20 minutes the activity was much less (figure 18).



Control perfusates and perfusate samples collected from 0 to 1, 1 to 2, 2 to 3, 3 to 4, and 4 to 6 minutes were incubated at 37°C for 10 minutes with 0.5 ml dog plasma pseudoglobulin. These results (table 14 and figure 19) show a rapid and considerable rise of bradykinin-forming capacity after shock. This effect disappeared in about five minutes. The first minute after challenge was the most active period. In one experiment where half-minute

samples were collected, the activity was greatest during the latter half of the first minute.

Time in minutes					
Control	0 - 1	1 - 2	2 - 3	3 - 4	4 - 6
14.0	111*	21	8	20	21
6	112	35	18	16	11
72	429	302	234	315	193
30	272	148	53	42	39
118	664	437	208	186	162

Table 14: Bradykinin-forming activity in isolated skin perfusate. Antigen was added at time 0.

* = the output was 43 ng in the first half-minute and 68 ng in the next half-minute.

As a control the same procedure was followed using unsensitised animals (table 15 and figure 20) and in other experiments sensitised animals were employed, but instead of antigen, histamine acid phosphate (≡ 5 µg histamine base) was administered.

Time in minutes					
Control	0 - 1	1 - 2	2 - 3	3 - 4	4 - 6
Unsensitised skin, antigen added:					
34	41	13	33	34	41
3	6	5	10	0	9
Sensitised skin, histamine added:					
25	37	38	25	29	19
14	12	10	11	6	12

Table 15: Bradykinin-forming capacity in isolated skin perfusate: control experiments.

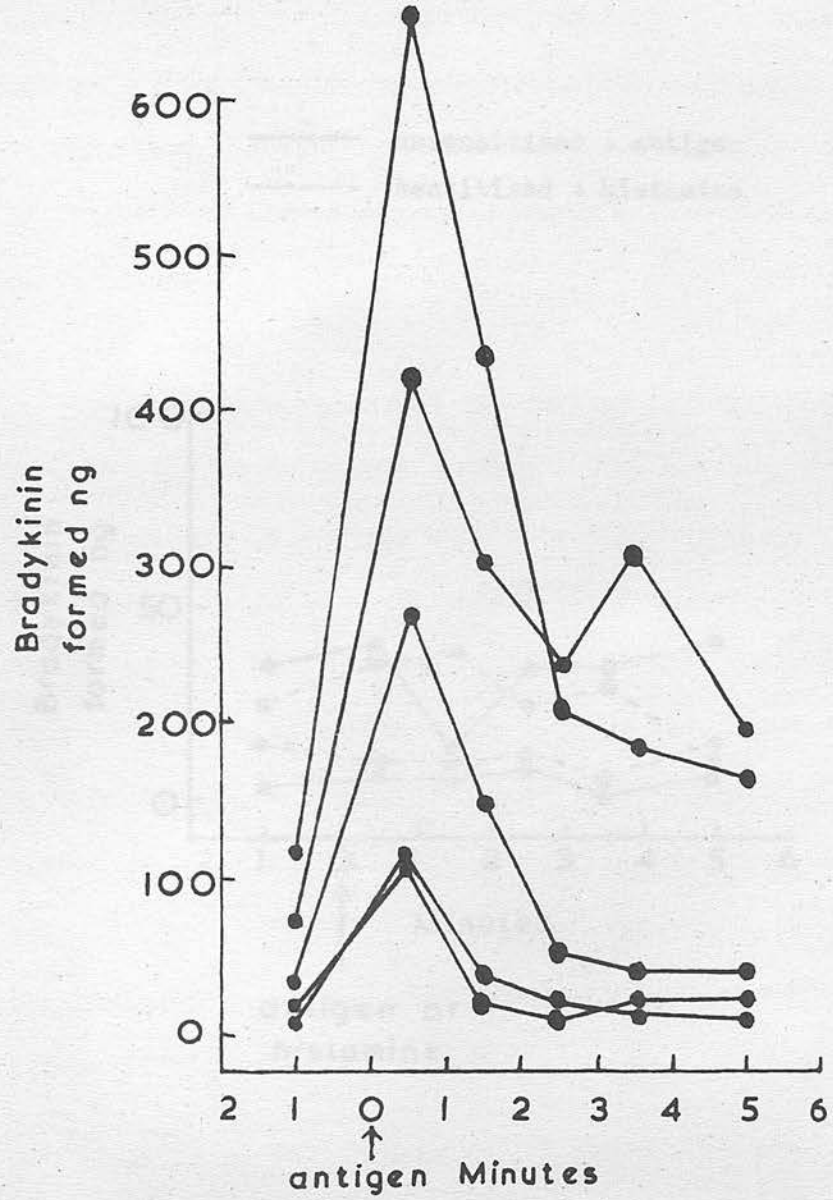


Fig. 19: Bradykinin-forming activity in guinea-pig skin perfusate after anaphylactic shock

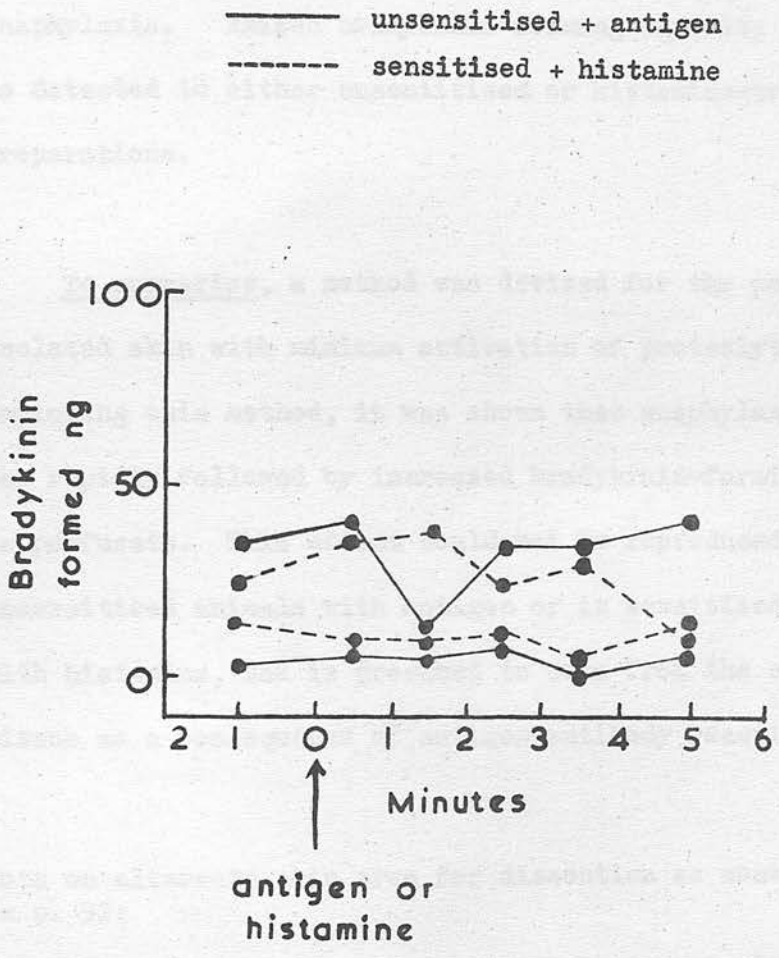


Fig. 20: Control experiments: estimation of bradykinin-forming activity in guinea-pig skin perfusate

These experiments were done to find out whether increased bradykinin-forming capacity could be induced by possible mechanical contractile effect of histamine released by anaphylaxis. Raised bradykinin-forming capacity could not be detected in either unsensitised or histamine-treated preparations.

To summarise, a method was devised for the perfusion of isolated skin with minimum activation of proteolytic enzymes. Employing this method, it was shown that anaphylaxis in vitro was rapidly followed by increased bradykinin-forming capacity in perfusate. This effect could not be reproduced in unsensitised animals with antigen or in sensitised animals with histamine, and is presumed to come from the cells of the tissue as a consequence of antigen-antibody reaction.

Note on alternate skin area for dissection as mentioned in p. 92:

*Other, though less convenient methods are also feasible. The cannulation of the same vessel could also be done through the external iliac artery; the approach was intra-abdominal, and vascular isolation was more difficult. Another area of the skin over the anterolateral aspect of the lower half of the thorax is also suitable for perfusion. In this case, the cannula, tied into the axillary or brachial artery, perfuses the branch of the brachial artery supplying the area concerned. These vessels, however, are more fragile and less suitable than the superficial circumflex iliac artery.

SECTION VI

BRADYKININ FORMATION DURING ANAPHYLAXIS IN SENSITISED GUINEA-PIG LUNG

BRADYKININ FORMATION DURING ANAPHYLAXIS IN SENSITISED GUINEA-PIG LUNG

The experiments described in this section were carried out to study the possible role of the lungs in the formation of bradykinin during anaphylaxis in guinea-pig.

Perfusion of blood-free sensitised lungs was carried out as described under "Methods and Material". Anaphylaxis was induced by adding 2 mg crystalline egg albumin in 0.2 ml warm saline to the perfusion fluid as it entered the lung. effluent. The collected perfusate was kept at 4°C until used the same day.

When perfusate collected before and after anaphylaxis was tested on rat uterus, no significant activity was seen in either. Occasionally, perfusate collected over the first minute after shock contracted the rat uterus only at concentrations as high as x 2 dilution in the organ bath. In such cases, the bradykinin-like activity in the entire sample was of the order of 1 ng, and this was held to be insignificant.

The absence of bradykinin in the perfusate from a blood-free organ did not exclude the presence of bradykinin-forming activity. This was investigated by incubating the perfusate with pseudoglobulin. Perfusate obtained during the two minutes before shock, and periods during 0-1, 1-2, 2-3 and 3-4 minutes after shock were incubated with heated dog plasma pseudoglobulin equivalent to 0.5 ml plasma, in 0.5 ml saline. After incubation at 37°C, the mixture was placed in a boiling water bath for 4 or 5

minutes to stop the reaction, cooled and stored at -10°C until assayed on rat uterus.

The optimum period of incubation of the reaction mixture was first examined by withdrawing aliquots from the incubation mixture at different times. The amount of bradykinin present was maximum at 10-15 minutes, after which this amount decreased (figure 21).

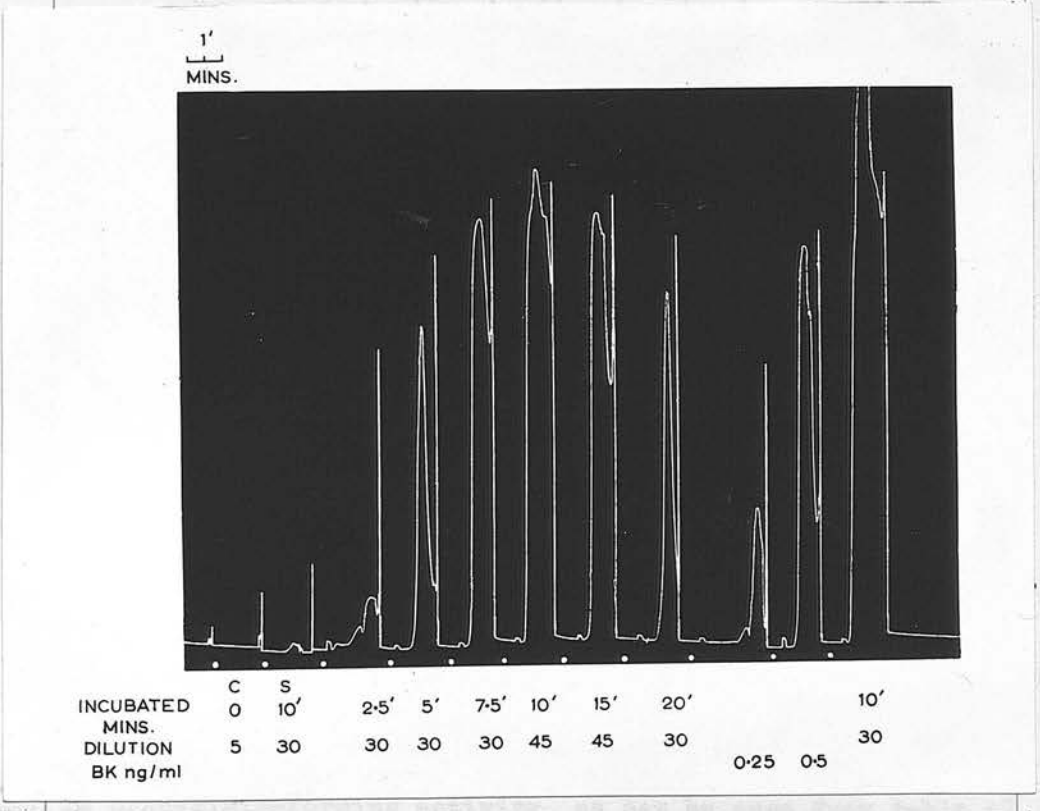


Fig. 21: Optimum period of incubation of post-shock lung perfusate with pseudoglobulin substrate: Rat uterus; dilutions of aliquots drawn at different intervals shown. C = control (not incubated); S = substrate; BK= bradykinin.

Substrate incubated in Tyrode, as a control, contained some bradykinin; this was taken into account when calculating the amount of bradykinin formed by the various samples. The amount of bradykinin formed on incubation for 10 minutes was calculated for control samples, as well

as samples collected between 0-1, 1-2, 2-3 and 3-4 minutes after anaphylaxis are shown in table 16 and figure 22.

Time in minutes				
Control	0 - 1	1 - 2	2 - 3	3 - 4
12	48	17	18	8
12	61	8	0	1
14	48	11	1	3
25	66	17	13	14

Table 16: Bradykinin-forming capacity of isolated lung perfusate; antigen was added at 0 minute.

It is evident that there is a sharp rise in the bradykinin-forming capacity of the perfusate in the first minute after shock, and a return to control level after 2-3 minutes.

For control, similar experiments were done with antigen in lungs from unsensitised animals; furthermore, in order to investigate whether these results were due to the mechanical effects of bronchoconstriction, 10 µg histamine instead of antigen was injected into sensitised perfused lung. Neither kind of experiment showed a rise in bradykinin-forming activity, as can be seen from table 17 and figure 23.

Time in minutes				
Control	0 - 1	1 - 2	2 - 3	3 - 4
Unsensitised lung, antigen added:				
7	3	3	7	6
11	13	5	11	11
Sensitised lung, histamine added:				
3	3	3	5	3
9	5	4	4	4

Table 17: Bradykinin-forming capacity in perfusate from isolated lungs; control experiments.

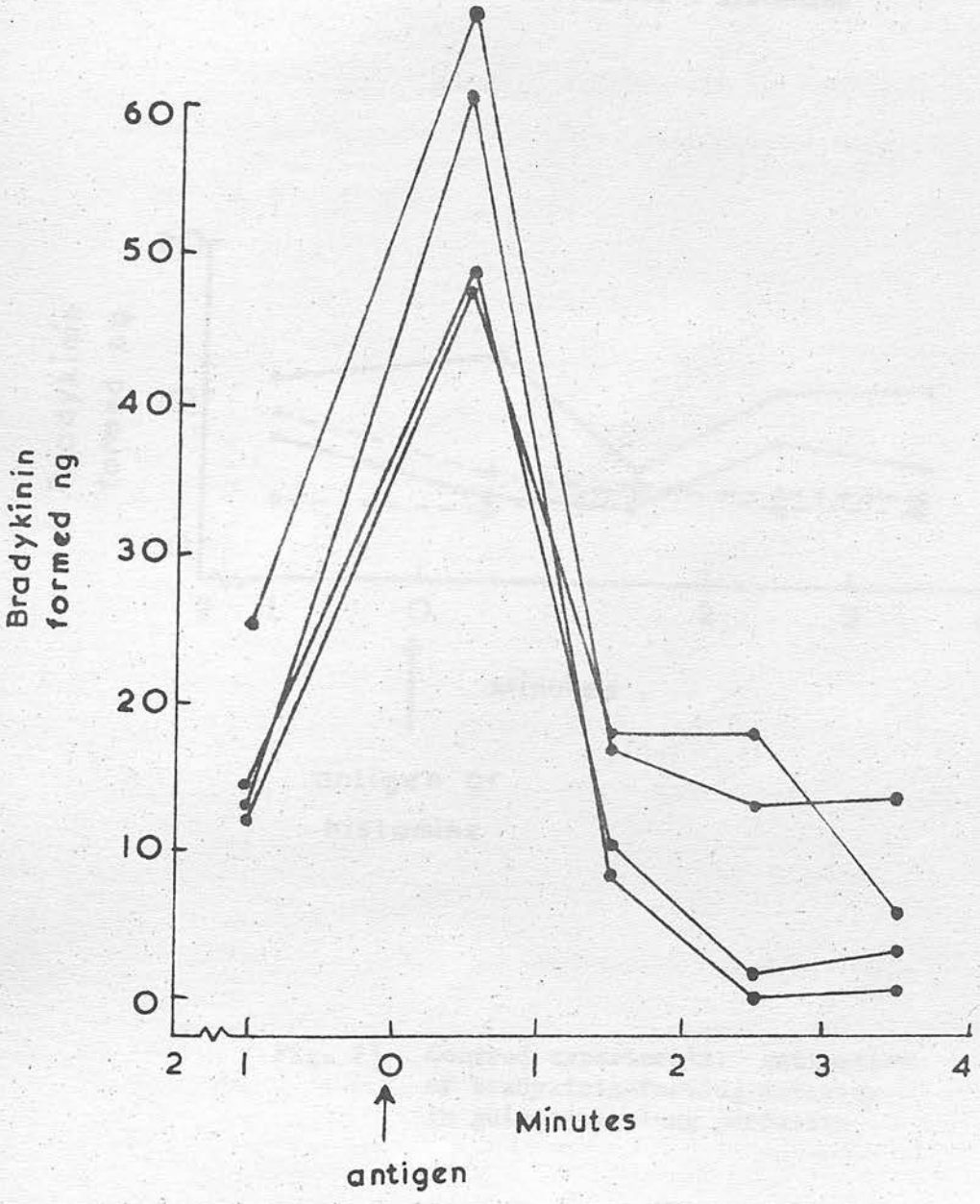


Fig. 22: Bradykinin-forming activity in guinea-pig lung perfusate after anaphylactic shock

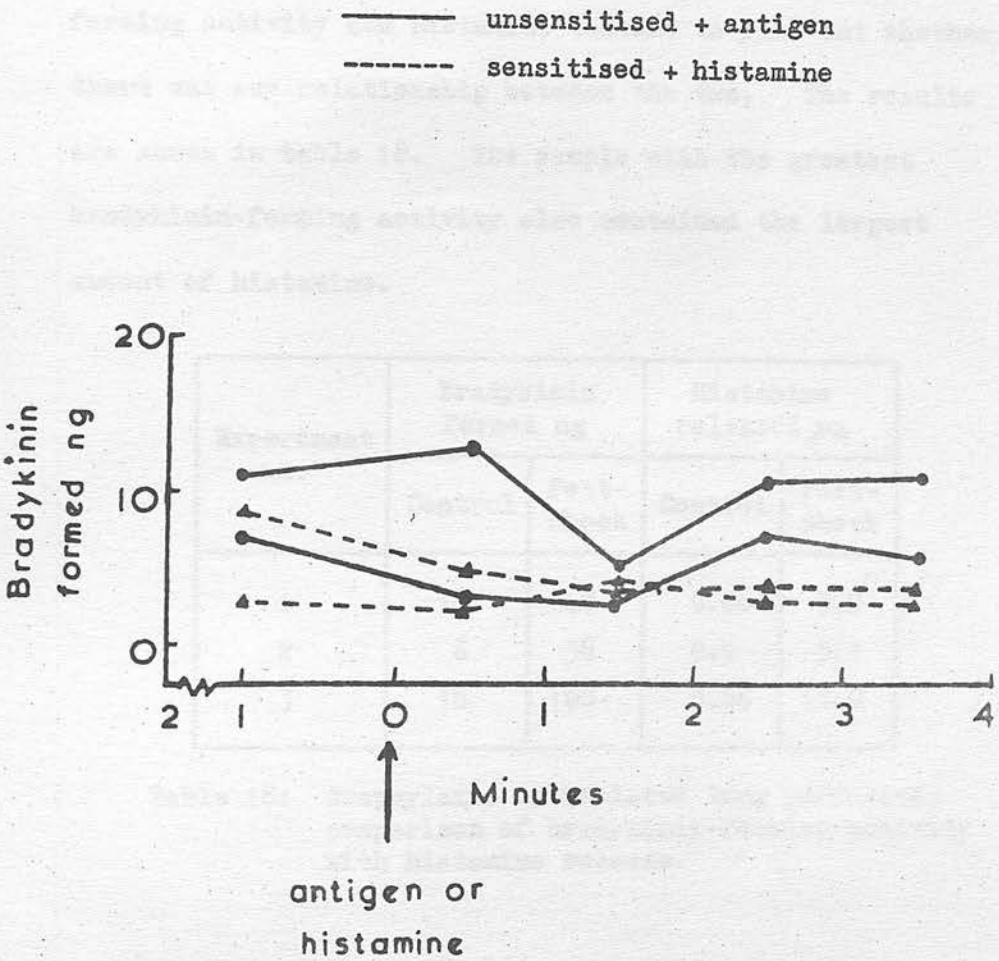


Fig. 23: Control experiments: estimation of bradykinin-forming activity in guinea-pig lung perfusate

Comparison of bradykinin-forming activity with histamine release:

In three experiments, sensitised lungs were perfused, and perfusate collected for 75 seconds before and after shock. These samples were estimated for both bradykinin-forming activity and histamine content to find out whether there was any relationship between the two. The results are shown in table 18. The sample with the greatest bradykinin-forming activity also contained the largest amount of histamine.

Experiment no.	Bradykinin formed ng		Histamine released µg	
	Control	Post-shock	Control	Post-shock
1	10	47	0.06	9.8
2	6	38	0.1	5.1
3	18	100	0.06	11.8

Table 18: Anaphylaxis in isolated lung perfusion; comparison of bradykinin-forming activity with histamine release.

Time-course with fresh guinea-pig plasma as substrate:

In order to correlate the findings <u>in vitro</u> described in this section with the course of events <u>in vivo</u> in guinea-pig, post-shock lung perfusate was incubated in polythene for different periods at 37°C with heparinised non-activated guinea-pig plasma as substrate (fig. 23A). It was found that the amount of bradykinin was highest after 2-3 minutes.

Fig. 23A: Optimum period of incubation of post-shock lung perfusate with fresh guinea-pig plasma. All incubation samples diluted x 15. BK = bradykinin; P3 = plasma incubated for 3 minutes; PC3 = plasma + control perfusate incubated for 3 minutes.

(Facing p. 107)

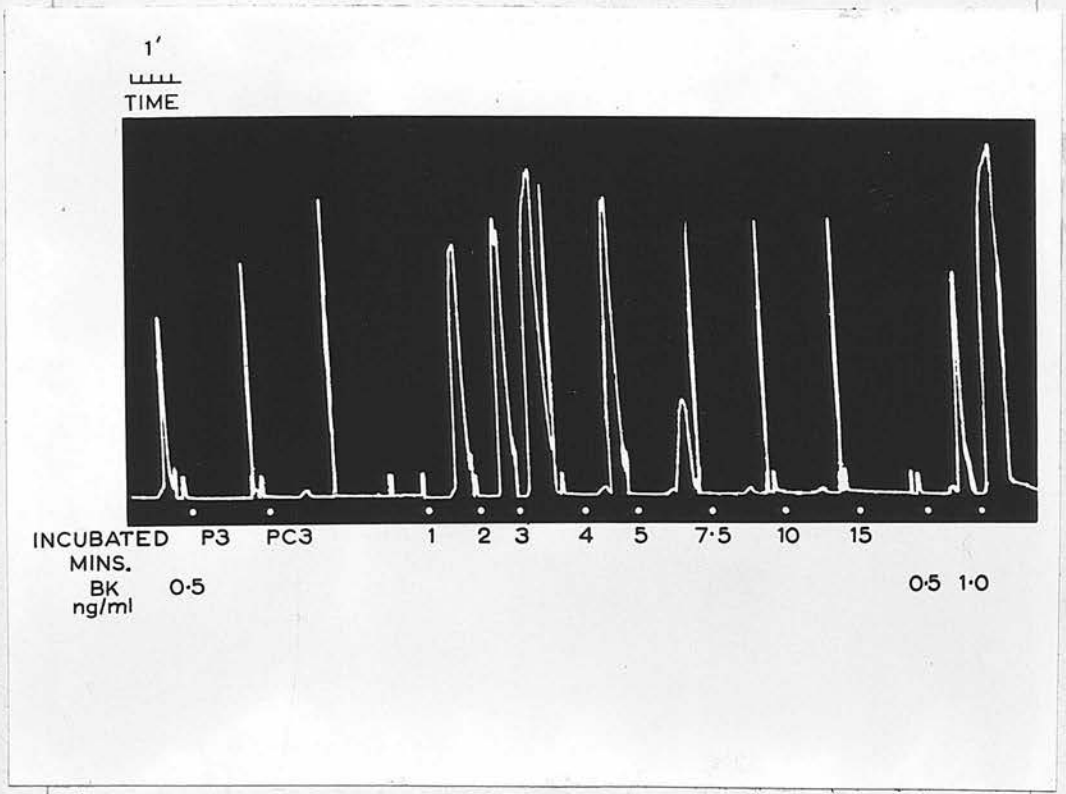


Fig. 23A

To summarise, experiments described in this section showed that anaphylaxis in guinea-pig lung in vitro is followed by a rapid and short-lived rise in bradykinin-forming activity in the perfusate. These effects could not be reproduced with antigen in unsensitised animals or with histamine in sensitised animals. The speed of bradykinin formation by the post-shock perfusate, when fresh guinea-pig plasma is used as substrate, is in accord with the time course of anaphylaxis in vivo. There appears to be some correspondence between the release of histamine and bradykinin-forming activity after shock.

SECTION VII

INVESTIGATION OF THE NATURE OF THE BRADYKININ-FORMING ACTIVITY OF ANAPHYLACTIC GUINEA-PIG LUNG PERFUSATE

INVESTIGATION OF THE NATURE OF ACTIVITY OF ANAPHYLACTIC GUINEA-PIG LUNG PERFUSATE

The experiments described in this section were done to find out the effect, on the bradykinin-forming activity in anaphylactic lung perfusate, of: (a) Heat, (b) pH, (c) ethylenediamine tetra-acetic acid, (d) soya bean trypsin inhibitor, and (e) hexadimethrine bromide.

In these experiments, the post-shock perfusate was usually collected for 75 seconds commencing 10 seconds after administration of antigen, and aliquots (usually 1 ml) of treated or untreated perfusate were compared. Sometimes, when several aliquots were required, samples from two or three animals were pooled.

Heat:

Aliquots were drawn from the same sample, one was kept untreated (as control); others were heated at 56°C for 30 or 60 minutes, or at 100°C for $2\frac{1}{2}$, 5, 10, 15 or 30 minutes, cooled, and then incubated with substrate for 10 minutes. The bradykinin formed was estimated on rat uterus. These results are shown in table 19. At 56°C more than half of the activity disappears after 60 minutes, whilst at 100°C some loss of activity is evident after five minutes and after 15 minutes most of the activity is lost.

Temp. °C.	% reduction in activity, taking unheated sample as 100%					
	Duration of heating in minutes					
	2	5	10	15	30	60
100°	13.5	30	44.8	82.1	90.1	
100°				96.0	96.1	
56°					35.6	58.1

Table 19: Effect of heat on bradykinin-forming activity in post-shock lung perfusate.

pH:

Equal aliquots of perfusate substrate mixture were adjusted with 0.06 M trishydroxymethylaminomethane ("tris") buffer to pH values between 5.7 and 8.0. After incubation for 10 minutes at 37°C, samples were boiled, cooled and assayed for estimation of the amount of bradykinin formed. The results are shown in table 20 and figure 24.

pH value	Amount of bradykinin ng calculated to be formed by perfusate collected during the first 75 sec after shock		
	Expt. I	Expt. II	Expt. III
5.7	-	21.3	0
6.0	92	100.8	0
6.25	-	142.1	46.8
6.5	342	475.3	132.7
6.75	502	624.4	182.7
7.0	427	363.0	155.1
7.5	347	117.0	96.5
8.0	108		69.9

Table 20: Effect of hydrogen-ion concentration on bradykinin-forming activity of post-shock lung perfusate.

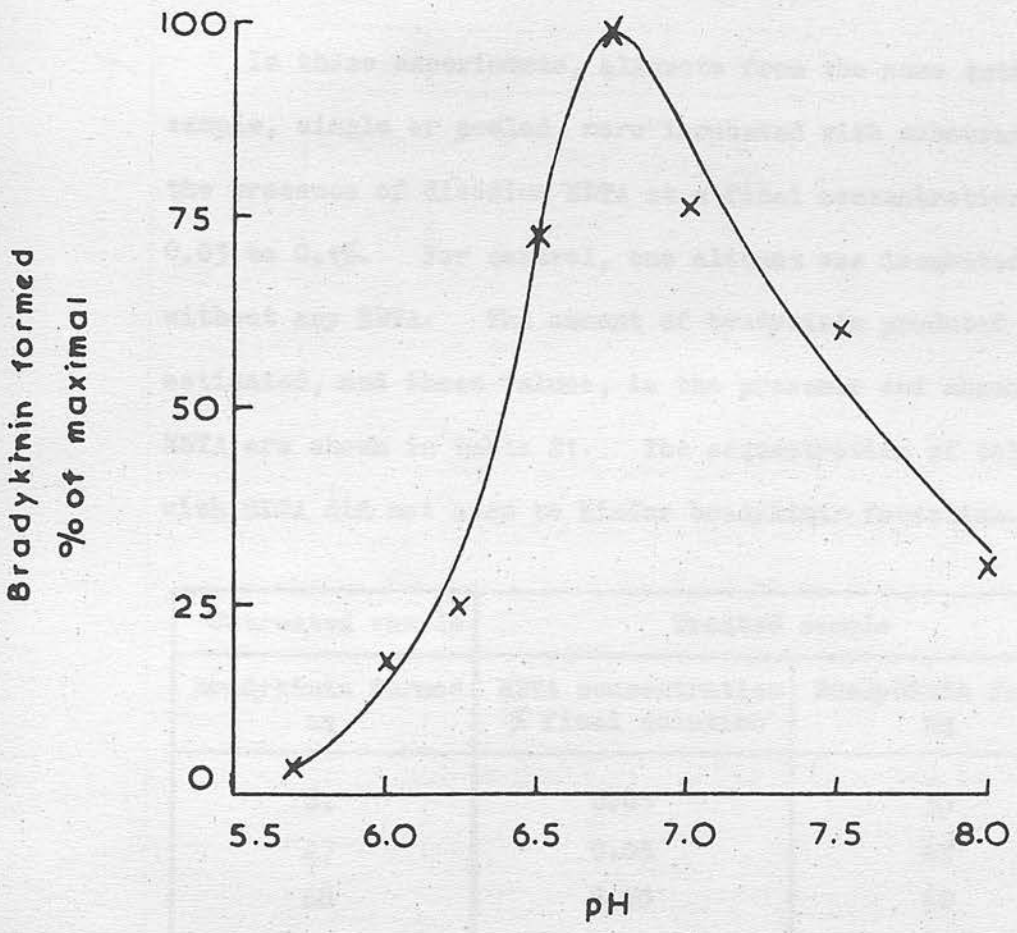


Fig. 24: Effect of pH on BKF. Average values are shown

In these experiments, optimum formation occurred at pH 6.75. Tests were included to ensure that the amounts of "tris" present in the mixture did not interfere with the response of rat uterus to bradykinin.

Ethylenediamine tetra-acetic acid (EDTA):

In these experiments, aliquots from the same active sample, single or pooled, were incubated with substrate in the presence of disodium EDTA at a final concentration 0.03 to 0.1%. For control, one aliquot was incubated without any EDTA. The amount of bradykinin produced was estimated, and these values, in the presence and absence of EDTA are shown in table 21. The sequestration of calcium with EDTA did not seem to hinder bradykinin formation.

Untreated sample	Treated sample	
Bradykinin formed ng	EDTA concentration % final solution	Bradykinin formed ng
61	0.03	53
47	0.05	49
68	0.10	60

Table 21: Effect of EDTA on bradykinin formation by post-shock lung perfusate.

Soya bean trypsin inhibitor:

Similarly, aliquots of anaphylactic perfusate were incubated in the presence or absence of soya bean trypsin inhibitor (2×10^{-4} g/ml final concentration). The

results are given in table 22. It was ascertained that the amount of soya bean trypsin inhibitor did not interfere with the bioassay. The results indicate that bradykinin formation under these circumstances is greatly inhibited by soya bean trypsin inhibitor.

Bradykinin formed by post-shock sample	
Untreated	SBTI-treated, final conc. 2×10^{-4} g/ml
47.0	8.8
157	- 14.2
40.3	- 8

Table 22: Effect of SBTI on bradykinin-forming activity of lung perfusate. Amounts in ng. Negative values result from subtraction of the control.

Hexadimethrine bromide (antagonist of heparin):

Similarly, aliquots were incubated in the presence or absence of hexadimethrine bromide. The results, presented in table 23, do not indicate any marked inhibition in the concentrations employed.

Bradykinin formed by post-shock sample			
Untreated	Hexadimethrine bromide-treated, final conc. g/ml		
	5×10^{-6}	10^{-4}	10^{-3}
24.5	20.3		
40		39	42

Table 23: Effect of hexadimethrine bromide on bradykinin-forming activity of lung perfusate. Amounts in ng.

To summarise, experiments in this section indicated that bradykinin-forming activity in anaphylactic lung perfusate was comparatively resistant to heat but could be destroyed by heating, had an optimum pH between 6.5 and 7.0, and a sharp cut-off at about 6.0. It was suppressed by soya bean trypsin inhibitor, but not by hexadimethrine bromide or by disodium ethylenediamine tetra-acetic acid, sufficient to remove most divalent cations including Ca^{++} and Mg^{++}.

SECTION VIII

KININASE ACTIVITY IN GUINEA-PIG LUNG PERFUSATE

KININASE ACTIVITY IN GUINEA-PIG LUNG PERFUSATE

The experiments described in this section were begun to see if kininase (bradykinin-destroying) activity was present together with the forming activity in guinea-pig lung perfusate. When such activity was found, further experiments were done to investigate the effects of (a) incubation period, (b) heat, (c) pH, (d) disodium EDTA, (e) hexadimethrine bromide, and (f) soya bean trypsin inhibitor on this kininase activity.

In order to estimate kininase activity, 1 ml of saline containing 100 ng synthetic bradykinin, was incubated with a sample of perfusate usually representing 15 seconds perfusion. After incubation, the reaction mixture was boiled in a water bath for 4-5 minutes to stop the reaction, cooled in ice and assayed against bradykinin on rat uterus the same day or stored at -10°C. The difference between the original amount and the amount left in the reaction mixture indicated the amount destroyed. Preliminary experiments showed that considerable kininase activity was present in the perfusate.

Period of incubation:

Mixtures of lung perfusate and synthetic bradykinin were incubated and aliquots were withdrawn at various

intervals from $2\frac{1}{2}$ to 40 minutes. The amount of bradykinin destroyed was calculated as a percentage of the original amount present. The results are shown in table 24 and figure 25. It can be seen that progressively greater proportions of bradykinin were destroyed with increasing periods of incubation, until after 40 minutes, only about 10% was left.

Expt. no.	Bradykinin ng destroyed (out of 100 ng) by various periods of incubation				
	2.5 mins.	5 mins.	10 mins.	20 mins.	40 mins.
1		6.4		52.9	
2			41.9	61.4	65.9
3		52.0	70	76	
4	9.5	20.0	63.3	75.5	
5			50.0	74.0	92.2
6			44.6	83.2	93.8
Mean % loss	9.5	26.1	54.0	70.5	84.0

Table 24: The time-course of bradykinin destruction by kininase activity in guinea-pig lung perfusate.

Heat:

In each experiment, aliquots representing 15 second perfusion were taken from a single perfusate sample collected during 75 seconds. The untreated (control) sample was kept at room temperature (18°C). Others were heated at 56°C from 5 to 30 minutes, or at 100°C from 2 to 15 minutes. After heating the samples were cooled quickly

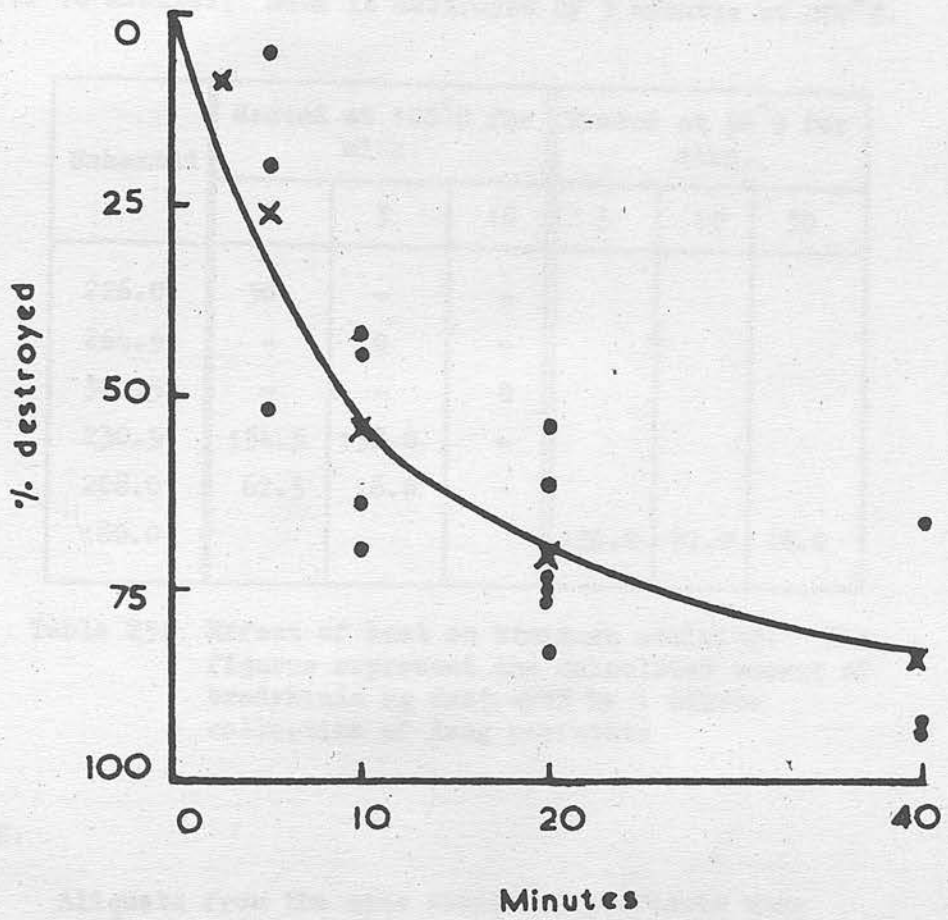


Fig. 25 : Time-course of bradykinin-destruction by kininase in lung perfusate

to room temperature. The control and heated samples were incubated with 100 ng bradykinin, and kininase activity expressed in terms of the amount destroyed. The results are shown in table 25. Kininase activity was barely detectable after heating at 56°C for 30 minutes or at 100°C for 10 minutes; much is destroyed by 3 minutes at 100°C.

Unheated	Heated at 100°C for mins.			Heated at 56°C for mins.		
	2	3	10	5	10	30
226.0	30	-	-			
264.5	-	0	-			
322.5	-	-	0			
230.5	154.5	138.0	-			
208.0	62.5	16.0	-			
180.0				126.0	92.0	16.0

Table 25: Effect of heat on kininase activity. The figures represent the calculated amount of bradykinin ng destroyed by 1 minute collection of lung perfusate

pH:

Aliquots from the same sample of perfusate were incubated at pH values, from 5.7 to 8.0, maintained by 0.03 M phosphate buffer. The kininase activity at different pH values, expressed in terms of bradykinin destroyed, are shown in table 26 and figure 26. The pH value for optimum kininase activity was between 7.0 and 7.5, and the activity was very greatly reduced by small changes of pH on either side.

pH	Bradykinin destroyed					
	Expt. I	Expt. II	Expt. III	Expt. IV	Expt. V	Expt. VI
5.7	3.4					
6.4					47.8	27.0
6.6					60.2	45.3
6.8			3.0	36.0	67.4	65.6
7.0	21.0	37.7	5.8	76.4	73.2	83.0
7.1			59.2	83.8		
7.2		54.2	51.6	87.4	90.3	73.4
7.3			52.6	88.4		
7.4		27.4	41.0	81.8		
7.6		16.8		75.4		
8.0	12.0					

Table 26: The effect of pH on kininase activity. The figures represent ng destroyed, out of 100 ng bradykinin by perfusate collected over 15 seconds.

EDTA:

Bradykinin was incubated with perfusate in the presence and absence of EDTA (final concentration 2 x 10^{-4} to 10^{-3} g/ml). The mixture was incubated for 20 minutes, and compared with control samples. The findings are shown in table 27. Very little inhibition occurs.

Hexadimethrine bromide and soya bean trypsin inhibitor (SBTI):

1 ml Tyrode containing 100 ng bradykinin was added to 1 ml lung perfusate, adjusted with Tyrode to represent 15

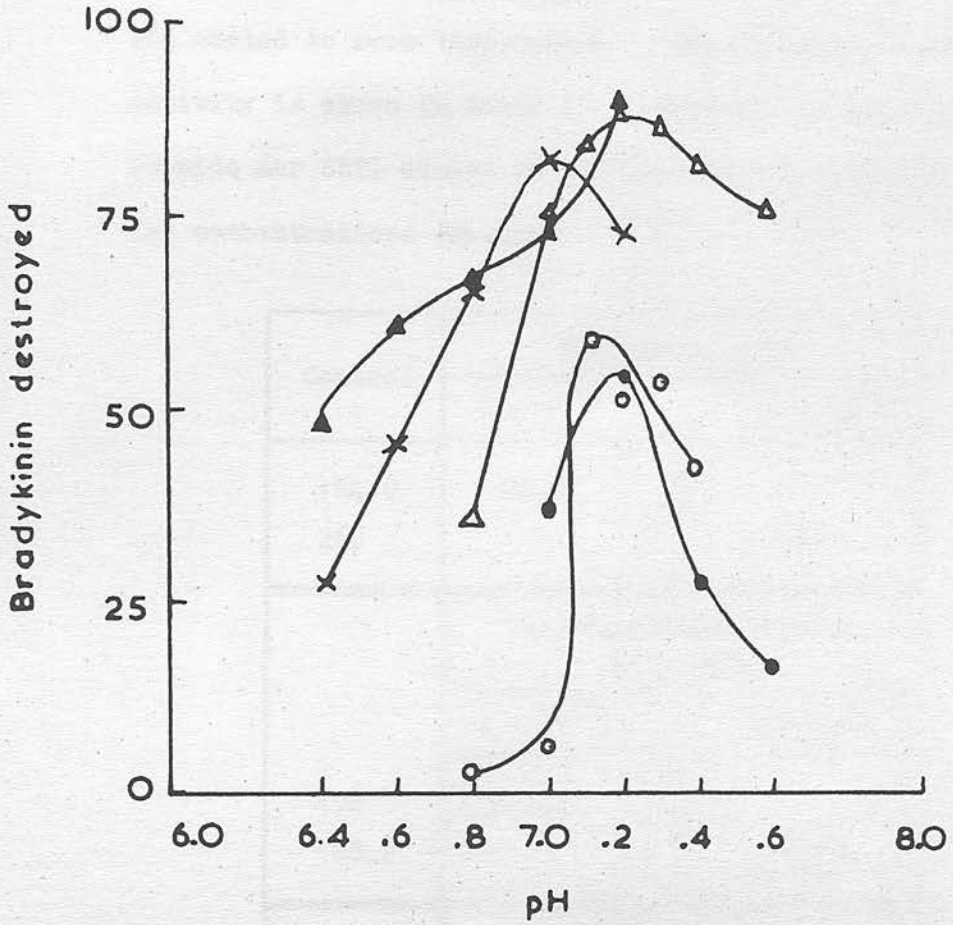


Fig. 26: Effect of pH on kininase activity. The ordinate shows the amount destroyed out of 100 ng when incubated with perfusate collected during 15 seconds. The different curves represent individual experiments.

or 20 second collection. To this mixture, either 1 ml Tyrode (to obtain control incubate) or SBTI (to give final concentration of 2 to 5 x 10^{-4} g/ml) or hexadimethrine bromide (final concentration 5 x 10^{-6} g/ml) was added. After incubation for 20 minutes, the samples were boiled and cooled to room temperature. The estimated kininase activity is shown in table 27. Neither hexadimethrine bromide nor SBTI seemed to inhibit kininase activity in the concentrations employed.

Control	SBTI conc. g/ml	
	2 x 10^{-4}	5 x 10^{-4}
164.8 262	166.6	260
	Hexadimethrine bromide conc. g/ml	
	2 x 10^{-4}	3.3 x 10^{-4}
178 168.2	175.5	151.4
	EDTA conc. g/ml		
	2 x 10^{-4}	3.3 x 10^{-4}	10^{-3}
178 168.2 163.4	178	121.4	158

Table 27: Effect of soya bean trypsin inhibitor (SBTI), hexadimethrine bromide and EDTA on kininase activity of lung perfusate. The concentrations shown refer to the final concentration in the reaction mixture. Other figures represent ngs bradykinin calculated to be destroyed by perfusate collected over one minute.

The amounts of kininase present in the effluent during the course of anaphylaxis in perfused guinea-pig lung:

Kininase activity was estimated in perfusate samples collected before and at various times after challenge. The findings are shown in table 28, and figure 27. Anaphylaxis did not seem to produce any significant alteration of kininase activity which is apparently present in normal tissue and spontaneously released during perfusion.

Time in minutes after shock	Experiment no.				
	1	2	3	4	5
Control	369	146	244	311	215
0 to 1	320	154	270	240	219
1 to 3	352	164			
3 to 5	329	158			
5 to 7		165			
5 to 10	374				
10 to 15	382				

Table 28: Amount of bradykinin destroyed on incubation for 20 minutes with perfusate obtained in one minute, during the period shown. Amounts in ng.

To summarise, experiments described in this section indicate that blood-free guinea-pig lung perfusate has considerable kininase activity, not appreciably altered by anaphylaxis. The kininase activity is thermolabile, has an optimum pH level between 7.0 and 7.5, and is very little affected by EDTA. Hexadimethrine bromide or soya bean trypsin inhibitor could not be shown to have any noticeable effect.

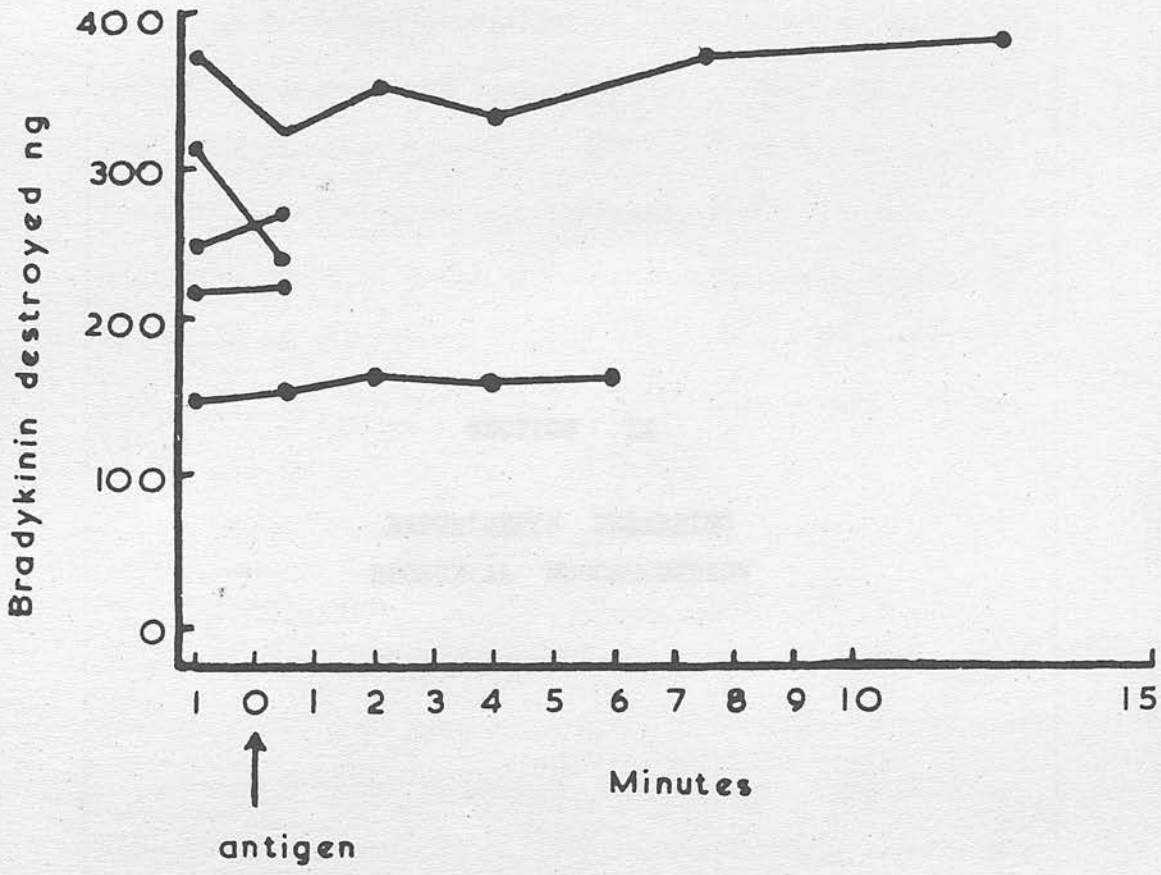


Fig. 27: Effect of anaphylactic shock on kininase activity in lung perfusate. Figures refer to the amount of bradykinin calculated to be destroyed by perfusate collected during one minute.

SECTION IX

EXPERIMENTS REGARDING BRONCHIAL MUCOSECRETION

BRONCHIAL MUCOSECRETION

The experiments in this section were done to investigate whether bradykinin increases bronchial mucosecretion.

As there are no standard methods for estimation of increased bronchial mucosecretion, the following method was adopted after some preliminary trials. This was based on the principle that in an isolated lung with the bronchial tree filled with physiological fluid, bronchial mucosecretion would be reflected in the mucoprotein content of the fluid in the lung. Mixing would result from rhythmic inflation, and the movement of fluid would permit sampling on the addition of drugs.

A glass chamber was set up as shown in figure 28 and made airtight with bungs A and B. Circulation of water at 37°C through its double walls kept it warm. A glass cannula about 5 cm long was passed through the centre of A about 1 cm into the chamber. A glass tube D was passed through another hole in A, and was connected to a three-way glass tap which led to compressed air and vacuum respectively. D was also connected with a mercury manometer.

A guinea-pig 300-400 g was killed by breaking the neck and the lungs removed along with the trachea. The cannula was tied into the trachea, and the preparation was thus suspended in the closed chamber.

20-25 ml Tyrode was introduced into the lungs through

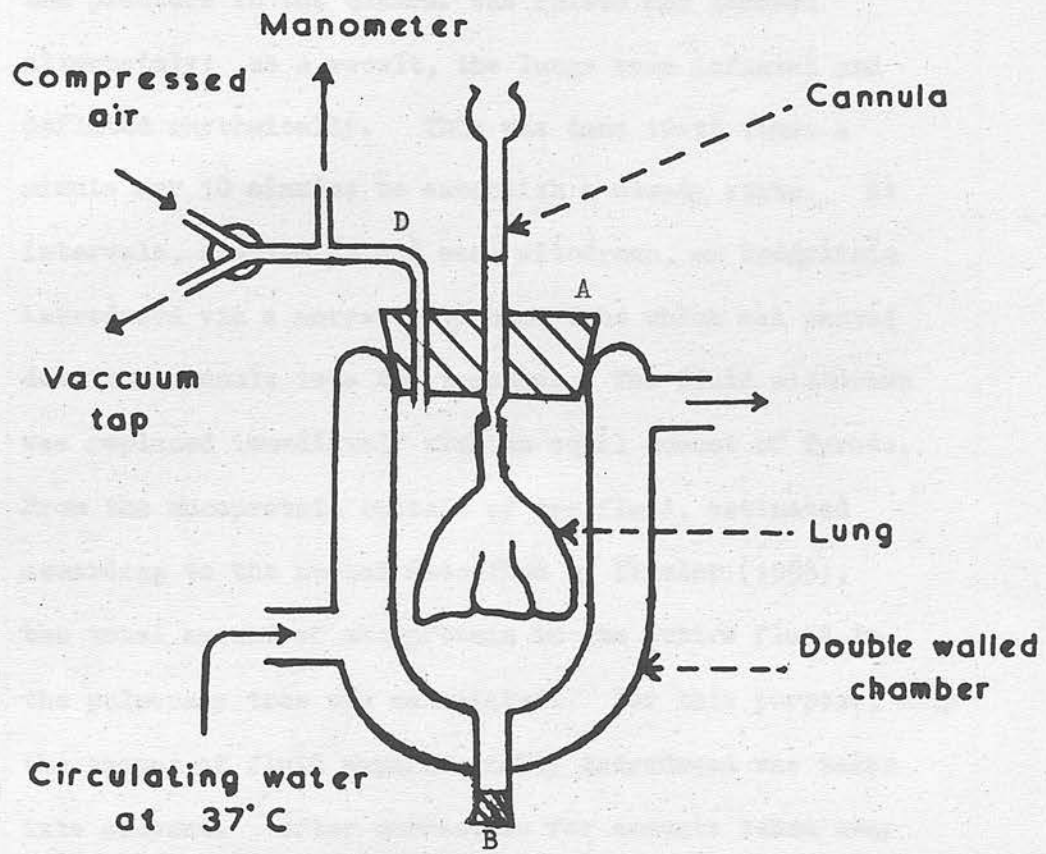


Fig. 28: Assembly for estimation of bronchial mucosecretion. The manometer records both positive and negative pressure. An adjustable valve for limiting both positive and negative pressure can be attached to a side branch (not shown) of tube D.

the tracheal cannula. It was found necessary to introduce this amount somewhat slowly to achieve proper filling up of the lung. Using air pressure or vacuum, the pressure in the chamber was raised and lowered alternately; as a result, the lungs were inflated and deflated rhythmically. This was done 10-15 times a minute for 10 minutes to establish a steady state. At intervals, samples (2 ml) were withdrawn, or bradykinin introduced via a narrow polythene tube which was passed down the cannula into the trachea. The fluid withdrawn was replaced immediately with an equal amount of Tyrode. From the mucoprotein content of the fluid, estimated according to the method described by Winzler (1955), the total amount of mucoprotein in the entire fluid in the pulmonary tree was calculated. For this purpose, only the amount of fluid experimentally introduced was taken into account. After correction for amounts taken away in successive samples, the total amount of mucoprotein present in the system was calculated in control and post-bradykinin samples. The results are shown in table 28 and figure 29. The rise in mucoprotein content in post-bradykinin samples as compared with successive control samples, is at best equivocal.

Histological confirmation of these results was attempted. Slices of normal lungs, and lungs from guinea-pigs 30 minutes after i.v. administration of 0.5 and 1.0 µg/kg bradykinin, were removed, fixed in Susa

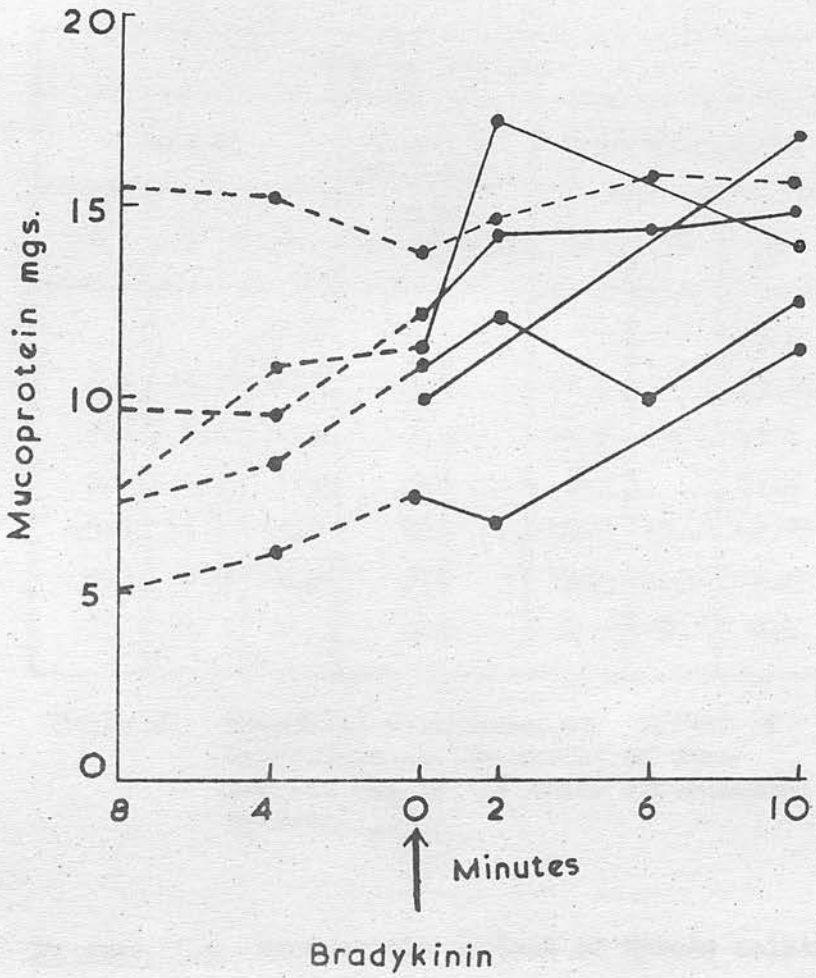


Fig. 29: Amount of mucoprotein estimated to be present in bathing fluid in isolated lung; interrupted lines represent estimations before coming in contact with bradykinin

solution and stained with Mayer's haematoxylin and counter-stained with alcian blue (by courtesy of Mr Peter Eyre of the Royal Dick Veterinary College, Edinburgh). The counterstain which would have brought out any mucus, failed to show any significant difference between normal and bradykinin-administered samples.

Time in minutes						
Control			Conc. of bradykinin ng/ml in system	Post-bradykinin		
0	4	8		10	14	18
		10.0	25			17.2
7.7	10.8	11.5	2.5	17.3		13.96
7.5	8.4	11.0	0.9	12.3	9.9	12.6
5.1	6.0	7.5	2.5	6.8		11.4
15.5	15.2	13.9	nil	14.7	15.7	15.7
9.7	9.6	12.2	3.0	14.4	14.2	14.9
0	0	0	2.5	0	0	6.4

Table 28: Bronchial mucosecretion. Effect of bradykinin on the amount of mucoprotein (mg in the whole experimental system).

To summarise, mucoprotein content of Tyrode solution kept in contact with the respiratory tree could not be shown to alter significantly after administration of bradykinin.

PART III

D I S C U S S I O N

DISCUSSION

In the course of the various sections presenting the results, some comments have been made on the aim and rationale of the experiments, and occasionally on the significance of the results. The purpose of this chapter is to discuss the various facets of the investigation and to integrate their significance.

The investigation was undertaken for a number of reasons. It has long been known that proteolytic activity in blood is associated with anaphylaxis. It is also known that increased amounts of urokinase occur in the urine of the guinea-pig after anaphylactic shock (Damgaard and Ungar, 1952). It seemed probable that such activated protease(s) might split bradykininogen, which is normally present in blood, and thus release bradykinin. Indeed, bradykinin is released in blood somewhat easily - by such simple measures as exposure to glass or dilution in physiological solutions. Furthermore, the main pharmacological effects of bradykinin, vasodilatation and increased capillary permeability, leading to hypotension, smooth muscle contraction, stimulation of sensory endings in the skin, and leucocyte migration, correspond to some of the features of anaphylaxis. This might account for certain phenomena which cannot be explained adequately on the basis of other active substances

known to be present during anaphylaxis. Finally, it was hoped that new data on the formation and destruction of bradykinin might shed light on its possible role in other pathological conditions.

A note on terminology is due here, particularly the use of the word "bradykinin" instead of the more general term "plasma kinin". Plasma kinin consists of bradykinin and/or other closely allied polypeptides with similar pharmacological properties. When this investigation was started, only bradykinin had been purified and synthesised; the structure of a second kinin (kallidin II) was determined much later. Hence, bradykinin was used as a standard in these investigations, and biological activity found to be bradykinin-like was measured against and expressed in terms of synthetic bradykinin. So the "bradykinin" referred to in the course of the present experiments should be taken to mean "bradykinin or polypeptides which cannot be distinguished from it pharmacologically". It is possible that part of the activity in the course of these experiments was due to kallidin and/or other kinins, but this would not materially change the significance of the present results. Even when other kinins are available in chemically pure form, preferably with specific antagonists, their separate and simultaneous estimation in biological material will be difficult; at present it is not possible.

There is only one report of the possible involvement of bradykinin in anaphylaxis (Beraldo, 1950), and subsequent work by Schachter (1956) and Armstrong et al. (1957) had

shown that the method used in 1950 was not satisfactory. Beraldo added the whole blood of anaphylactic dogs, taken "before and several minutes after shock", directly to an isolated guinea-pig ileum preparation in the presence of mepyramine. Seven out of 18 post-shock samples caused a slow contraction of the gut. The degree of activity present varied considerably, and some samples had to be incubated before any activity was found. On account of the wide variability and the small amount present, Beraldo suggested that bradykinin was unlikely to play any significant part in anaphylaxis. In 1950 it was not known that exposure to glass or dilution in physiological solution activated plasma to form kinins; nor had the kininase activity normally present in blood been realised. It is possible that glass-activation and/or dilution-activation could account for some of Beraldo's results. Glass-activation is the most likely explanation of samples which showed no activity "immediately after they were withdrawn", but where "activity could be observed after incubation of the blood samples", presumably in a glass container. Dilution-activation must also be considered since blood samples were put directly in the organ bath. Finally the amount of bradykinin present in the post-shock samples must have been influenced by kininase activity during the interval between the withdrawal of the sample and biological assay. No precautions are recorded, and this time-interval presumably varied in different experiments. Therefore in 1960 it seemed necessary to re-examine the whole matter

using methods designed to prevent changes consequent upon withdrawal of blood.

Bradykinin levels in blood had to be studied to examine the effect of anaphylaxis on bradykinin release *in vivo*. No method was available for the estimation of bradykinin in circulating blood, and such a method had to be developed first. In shed blood, formation and destruction of bradykinin proceed simultaneously. A suitable estimation method must ensure as far as possible that interfering enzyme activity is suppressed, and that bradykinin is neither formed nor destroyed spontaneously. In the present method advantage was taken of the fact that bradykinin is soluble in ethanol-water mixture, and interfering enzyme activity will be checked by 70 - 80% ethanol and also diminished by cold. Therefore arterial blood was run straight into cold ethanol so as to have approximately 80% ethanol in the final mixture. Whole blood rather than plasma was used to avoid bradykinin loss due to kininase activity during the 10-15 minutes required to separate plasma from whole blood. The use of whole blood resulted in the extract containing K^+ from the erythrocytes, but this did not lead to any serious difficulties in assay, except that it imposed a minimum degree of dilution of the samples (usually at least fivefold) before testing on the rat uterus. The difficulty can be partly obviated by reconstituting the dried material in saline instead of de Jalon solution. Errors due to K^+ are unlikely because of the different

time-course of the response of the uterus: the quick contraction of K^+ can be easily distinguished from the slower contractions due to bradykinin and there is little summation. The need for a certain amount of dilution lowers the limit of detection to levels of the order of 5 ng/ml blood. The experimental limitations imposed by the present method concerns only control samples and others of very low activity; due to the high sensitivity of the rat uterus, active samples were nearly always diluted far more than fivefold. Hence for the sake of simplicity, further purification was not attempted. If further purification is desired, various possibilities exist, e.g. ion exchangers, butanol extraction, or extraction with 95% ethanol in the presence of sodium chloride. In control samples, the amount of bradykinin present was usually below the threshold detectable by assay. After anaphylactic shock, however, in all cases the levels rose well above the threshold detectable. Thus the true increase will sometimes be slightly larger than that quoted in the results.

Acute anaphylactic shock produces a distinct but brief rise of bradykinin level in blood in all three species examined - guinea-pig, rat and rabbit. Clinical allergic conditions are often chronic in nature and perhaps from this point of view, intraperitoneally challenged guinea-pigs offer a better model than intravenously challenged animals. When antigen is slowly released from a depot, as with alum-adsorbed preparation from the guinea-pig, the sudden and usually fatal release of large quantities of histamine does

not occur. Under these circumstances, bradykinin is released over a long period. Such a gradual application of antigen would occur when pollen is inhaled or allergen comes in contact with the skin.

If bradykinin plays any part in anaphylaxis its formation and release should conform to a time-course consistent with the speed of events during anaphylactic shock in the animal studied. The present experiments showed that in acute shock the time taken to reach peak blood bradykinin level conformed roughly to the time taken for anaphylactic features to develop in the species examined. For example, in the guinea-pig, peak blood bradykinin level is reached in $2\frac{1}{2}$ minutes. The same time-pattern is seen when anaphylaxis is induced *in vitro* in perfused guinea-pig lung and skin; a high level of bradykinin forming activity appears in the first one or two minutes following challenge. When anaphylactic guinea-pig lung perfusate is incubated with the substrate it would normally act on *in vivo*, i.e. fresh guinea-pig plasma, the same time-pattern is seen. Under these conditions, maximal bradykinin formation occurs in 2 - 3 minutes, corresponding to a total delay after challenge *in vivo* of 3 - 4 minutes. These findings correspond in a general way to the speed of events seen during anaphylaxis *in vivo* in guinea-pig.

The fall of blood bradykinin level is presumably due to kininase activity. This is known to be present in blood, and as the present findings suggest, in tissue also.

The ubiquity of kininase is probably responsible for the comparatively quick reduction of the raised bradykinin level in blood, in case of intravenous challenge. It should also be noted that the hypotensive effect of intravenous bradykinin is short-lived; recently the biological half-life of bradykinin has been calculated as < 0.5 minutes in women (Saameli and Eskes, 1962).

The active principle in anaphylactic blood was identified as bradykinin-like by parallel quantitative assay, the effect on rat duodenum, and chymotrypsin digestion. Histamine, acetylcholine and 5-hydroxytryptamine could be excluded by the use of antagonists. The highest index of discrimination was 2.0, which is reasonable considering the comparatively crude nature of the extract but could indicate the presence of other kinins e.g. kallidin II. In rats made resistant to histamine and 5-hydroxytryptamine with antagonists, anaphylaxis lowers blood pressure; so does bradykinin. This indirect but supportive evidence suggests that bradykinin plays a part in the features of anaphylaxis. Taken with the rise of blood bradykinin level in rat during anaphylaxis, bradykinin release might explain the findings of Sanyal and West (1958) who found that rats could be severely shocked with antigen even when they were protected against histamine and 5-hydroxytryptamine with antagonists. This may also explain the finding of Brocklehurst, Humphrey and Perry (see later) that cutaneous manifestations in rat due to antigen-antibody reaction were largely mediated by something

other than histamine and 5-hydroxytryptamine.

The release of bradykinin in blood after anaphylaxis should lead to a depletion of its bradykininogen content: this was investigated. Experiments with trypsin-digested plasma (Schachter, 1956) gave a very poor yield, presumably due to kininase and perhaps anti-kininase activity of plasma. The other methods in which plasma is fractionated (Holdstock, Mathias and Schachter, 1957) to obtain preparations with better yields, are unsuitable for small samples (0.2- 0.5 ml plasma), not least because two dialysis procedures are involved. In the development of a suitable and simple method it was felt that the crucial procedure would be to suppress all enzymic activity as quickly and completely as possible. In the present method this was achieved by ice-cold ethanol in excess; full denaturation resulted from subsequent boiling. This method has a further advantage. Ethanol precipitates bradykininogen with other proteins, but as bradykinin is ethanol-soluble, examination of the supernatant permits a determination of the free bradykinin present in plasma in parallel with the estimation of bradykininogen from the precipitate. Diniz, Carvalho, Ryan and Rocha e Silva (1961) have described a method for estimation of bradykininogen in small samples. In this, denaturation is effected by boiling in dilute acetic acid. With this method, these workers found an average of 8.8 µg/ml plasma (range 7.9- 10) in the guinea-pig (bradykininogen expressed in terms of the amount of bradykinin formed on trypsin-

digestion). This is lower than the average value obtained by the present method (12.86 µg/ml, S.E. 1.9). In order to compare the efficiency of the two methods, bradykininogen content of aliquots from the same samples of guinea-pig plasma were compared in parallel employing the two methods. As table 30 shows, better yields were obtained with the method devised in the course of this work.

Bradykininogen ≡ bradykinin µg/ml plasma; estimated according to the	
Present method	Method of Diniz et al.*
19.1	10.2
23.0	14.8
15.7	11.4

Table 30: Comparison of bradykininogen yield from plasma by the present method and that of Diniz et al. (1961)

* The average bradykininogen value reported by these workers is ≡ bradykinin 8.8 µg/ml plasma

The very recent paper of Henriques, Picarelli and Oliveira (1962) shows that the enzymes in the plasma of certain species (horse) are in fact activated by incubation at pH 4, so that some loss of bradykininogen might occur during heating to 100°C. For comparison of control and post-shock samples, guinea-pigs were challenged intraperitoneally because with retarded and comparatively long-lasting shock, bradykininogen depletion was more likely to be detected. It soon became

evident that the amount of bradykininogen used up was far in excess of the amounts one would expect from the peak blood bradykinin levels. For example the depletion of bradykininogen is of the order of ≡ 6 µg bradykinin per ml plasma; this is about 50 times the peak bradykinin level found in blood (i.e. 50 ng/ml ≡ approximately 100 ng/ml plasma) during anaphylaxis. The difference is easily understood when one considers the high level of kininase activity normally present in tissue apart from that in the blood. The levels of bradykinin detected during anaphylaxis reflect a dynamic process; bradykininogen is split to release bradykinin, and simultaneously the bradykinin is being destroyed by kininase present in tissue and blood. When formation exceeds destruction, the level of free bradykinin in blood rises. Since it must be presumed that the rate of destruction will rise with increase in the concentration of bradykinin, the system tends to be self-limiting and high concentrations of bradykinin may never occur. The present results show that in retarded-type anaphylaxis large amounts of bradykininogen are lost. Therefore the gross amount of bradykinin formed is probably considerable; however, the net amount of bradykinin present in blood at any one time is small because of continuous destruction. Nevertheless, the low concentration is likely to be above threshold for many tissue responses, particularly that of raised vascular permeability, so the presence of bradykinin for long periods may account for some of the manifestations of allergy and

asthma.

The release of bradykinin, with concomitant depletion of bradykininogen led to an investigation of the site and mechanism of bradykinin formation following anaphylaxis. Two situations in which bradykinin may be formed were investigated: (a) challenge in vitro of shed blood, in the absence of tissue; and (b) challenge in vitro of tissue in the absence of blood. The role of histamine was also considered. In the first case, significant release of bradykinin could not be detected in the blood of either guinea-pig or rat. This would suggest that some factor from tissue is essential for anaphylactic bradykinin release in blood.

Since no bradykinin was released on challenge of shed blood, blood-free tissue was next examined. Skin and lung were chosen for this purpose for practical reasons and also because these tissues are of particular interest in allergic state.

During investigation of possible bradykinin formation in skin during anaphylaxis in vitro it is essential to avoid activation of proteases during experimental manipulations. The usual skin perfusion procedure (Feldberg and Paton, 1951) is not suitable since the use of a thermocautery is very likely to activate proteolytic enzymes; indeed, bradykinin is said to be released by damage of the skin by heat (Rocha e Silva and Antonio, 1960; Rocha e Silva and Rosenthal, 1961). The present preparation avoids these difficulties as far as possible. However, it has certain limitations.

While ink-injected specimens (figure 17) suggest good perfusion, the actual proportion of the total vascular bed which is actually being perfused will vary from preparation to preparation and perhaps in the same preparation from time to time. This factor, which will largely determine the extent of antigen-antibody reaction achieved, may explain the considerable variation in the level of the peak bradykinin-forming activity reached after anaphylaxis. The general pattern of response, however, remains the same in every experiment, i.e. a sharp rise in the first minute followed by decline over the next few minutes. In some experiments attempts were made to keep a high proportion of the vascular bed open by perfusing under greater pressure, but oedema appeared soon, and the preparations had to be abandoned. With the usual pressure head (150 cm water) oedema appears only after 45 to 60 minutes of perfusion.

The absence of bradykinin in the perfusate is not surprising, since bradykininogen is presumably absent in these blood-free preparations. These experiments indicate that anaphylaxis results in the rapid release of a substance(s) capable of forming bradykinin on incubation with bradykininogen. For easy reference, this will be referred to as BKF (Bradykinin Forming Factor). The extremely rapid rise in BKF level after anaphylaxis points to an almost explosive release, which is faster than that of SRS-A (Brocklehurst, 1960) and is of the same order as histamine. Hence the possibility was considered that BKF release was not initiated by the antigen-antibody reaction

itself, but by the attendant histamine release which might induce leakage of BKF from the tissue and thence into the blood vessels due to increased permeability. Since histamine administration did not increase BKF, its release cannot be dependent upon histamine, though the increased permeability due to histamine is probably an accessory factor. These findings differ from those of Edery and Lewis (1962b) who found that the increased bradykinin-forming capacity of lymph from a dog's hindlimb subjected to burning, trauma or reactive hyperaemia could be reproduced by histamine. These workers did not find any increase in BKF in the effluent from a perfused limb after injury. The experiments are different in many respects (e.g. species, organ and nature of injury) - quite apart from the presence of blood and route of collecting the active material. It may be that in the intact limb something akin to dilution-activation occurs when the vascular permeability alters, and histamine would then produce such an effect. In the anaphylactic reaction, the damage would extend progressively from the vascular supply to the other tissue, and very slowly to the outer epidermis, whereas in the experiments of Edery and Lewis (1962) the damage would originate extravascularly, and the sequence and probably the mechanism may be quite different. Edery and Lewis incubated the enzyme-containing material with substrate for two minutes, and do not mention whether longer incubation periods were also tried. The time of incubation may be important because in the present work it was found

that whereas the optimum time for incubation using fresh homologous plasma as substrate was about 2 minutes, the optimum time when heated dog plasma pseudoglobulin was used was about 10 minutes, and very little bradykinin is present after 2 minutes. These two substrates are comparable with those used by Edery and Lewis in the lymph collection and perfusate collection respectively.

Skin is known to contain proteases (Beloff and Peters, 1945; Neville-Jones and Peters, 1948). It has been reported that acetone-dried rat skin does not yield protease except with drastic procedures like extraction with potassium thiocyanate, and such thiocyanate extracts have been shown to form plasma kinin on incubation with suitable substrate (Lewis, 1959). An easier extraction procedure, with buffered KCl, was reported by Inderbitzin and Goetschmann (1959). They separated two enzymes from acetone-dried rat skin. One was comparatively thermostable and acted optimally in neutral or slightly alkaline solutions. The amount of this enzyme present in skin does not alter after occurrence of PCA (Passive cutaneous anaphylaxis) in skin. The other enzyme was more thermolabile and acted optimally at an acid pH. Skin previously subjected to PCA contains less of this enzyme than normal skin. These workers therefore suggested that increased capillary permeability produced in the rat by PCA is due to the release of a proteolytic enzyme. It is possible that this was a bradykinin-forming enzyme because the present work has shown loss of BKF into the perfusion fluid during

anaphylaxis and this is active in neutral or slightly active media. Inderbitzin and Goetschmann (1959) found that the increase in cutaneous capillary permeability in anaphylaxis is reduced by antiproteolytic agents. The nature of the protease was not established, but this work suggests that bradykinin-like active substances may be important.

The lung, as the shock organ in the guinea-pig was chosen for most of the experiments in vitro. The absence of bradykinin itself in the shocked lung perfusate confirmed the earlier observation of Brocklehurst (1958a), though it is only to be expected in a blood-free system. There was an explosive release of BKF after shock, the time-course being very similar to that of histamine. This distinguishes it from the time-course of SRS-A release as elicited by Brocklehurst (1960). The possibility that BKF release was due to the increase in permeability and contraction produced by histamine was considered, and excluded by the control experiments where histamine, but not antigen, was administered. In lung, as in skin, the permeability-increasing and contractile effect of histamine does not initiate BKF release, but probably promotes its effect by facilitating its contact with bradykininogen from blood.

The time-course of BKF release and the speed of action on plasma or lymph could produce bradykinin quickly enough to play a part in the guinea-pig even when collapse occurs within 3 or 4 minutes.

In the present investigation no attempts were made to

elucidate the mechanism of BKF release from lung. However, there is a rough correspondence in the amount of BKF and histamine released, as also the time-course of release. One may speculate that the steps leading from antigen-antibody reaction to histamine release and BKF release have a large part in common, simply involving sufficient cell damage to cause loss of histamine from mast cell granules or protease from lysosomes.

After the release of bradykinin in vivo was detected it was felt desirable to study this in different tissues and species. Rapp (1962) found that in the rat, antigen-antibody reaction in the peritoneal fluid results in the appearance of SRS-A. In these experiments, antibody is given intraperitoneally 4 hours before antigen. This procedure was followed, but even control samples had considerable bradykinin-like activity. The activity in post-shock samples was not sufficiently enhanced to permit any conclusions. When Tyrode was used instead of saline, control values were lower. This indicates that bradykinin is formed in rat under conditions which deviate only slightly from normal physiology; indeed, large amounts of bradykinin are formed in rat plasma comparatively easily (Rocha e Silva and Holzhacker, 1959). In the present experiments the usual precautions against spontaneous activity (siliconed glassware, storage on ice) were taken, but the interval between collection of the samples and their assay varied from a few minutes to about two hours; in every case control levels of bradykinin were too high to permit

satisfactory experiments. It might be instructive to apply the technique used by Rapp (1962) to the guinea-pig because the mast cells of this animal are known to be less easily destroyed.

It was necessary to obtain some information concerning the properties of BKF. The formation of bradykinin on incubation of BKF with pseudoglobulin substrate has been assumed to be enzymic, though it is still a little uncertain whether BKF is the enzyme itself or an activator of the precursor of a bradykinin-forming enzyme known to be present in the dog globulin substrate employed. This question can be settled only when pure bradykininogen is available, or at least partially purified bradykininogen, free from forming or destroying enzymes or their precursors. Soya bean trypsin inhibitor inhibits BKF activity, but as it inhibits kallikrein this does not necessarily prove that BKF itself is an enzyme. BKF is more heat-resistant than kininase, though the difference is not so marked as to permit the selective destruction of kininase while BKF is left intact. The question that BKF might be the same as the enzyme which forms bradykinin on glass-activation or dilution was considered (Armstrong et al., 1962). This question will have to be left open as the available evidence does not permit a firm conclusion. Hexadimethrine bromide inhibits the enzyme purified by Armstrong and her colleagues at a final concentration of 2 to 5 x 10^{-6} g/ml. To compare BKF with this enzyme, the effect of hexadimethrine bromide on BKF was examined. Final concentrations as high as

10^{-3} g/ml produced practically no reduction in BKF activity. This argues against BKF which comes from tissue being the same as the enzyme activated in plasma. However, it should be remembered that different substrates were used in these experiments (dog pseudoglobulin and human plasma) and the results therefore are not strictly comparable.

From the experiments described in section VII the best pH for bradykinin formation by BKF would appear to be approximately 6.75; this was not known at the time of the earlier experiments where the release of BKF was established. In these experiments pseudoglobulin was incubated with Tyrode perfusate, hence optimal conditions were not employed. Even under these circumstances, the pattern of response is quite clear.

The present findings on the effect of pH on BKF activity is interesting. A slightly acid milieu (pH value 6.5 to 7.0) is best for the release of bradykinin. It is probable that in bronchial asthma hypercapnia and the usually attendant emphysema may lead to respiratory acidosis and favour bradykinin formation. In other pathological conditions such as burn, trauma and reactive hyperaemia where bradykinin is suspected to play a role, the release of acid metabolites like lactic acid, CO_2 and phosphoric acid may lead to a local lowering of pH to 6.5 to 7.0 and thus favour bradykinin formation. At this pH kininase activity is considerably reduced. The net amount of bradykinin present is a balance between the simultaneous processes of formation and destruction. A slightly acid

pH, quite capable of being reached in tissue subjected to mild oxygen lack would therefore tend to raise the net level of bradykinin present by both increasing bradykinin formation and reducing destruction. It is of interest to recall that pus and some exudates are acidic in nature.

Because nothing is known yet of the substrate specificity of BKF, no opinion can be formed about possible relationship between BKF and the chymotrypsin-like enzyme which Austen and Brocklehurst (1961a) consider to be essential for anaphylactic release of histamine from guinea-pig lung; a priori there would seem to be no relationship because BKF would be expected to resemble trypsin rather than chymotrypsin.

The known occurrence of kininase activity in blood (Armstrong et al., 1955; Schachter, 1960) and the large turnover of bradykininogen in anaphylaxis, prompted tests for kininase activity in tissues about which little is known. Werle (1955) and Rocha e Silva (1955) have referred to kininase activity in liver and kidney, but presumably these tissues were not perfused free of blood, and it is difficult to know how much of the kininase activity detected was due to the blood present in the organs examined. As far as can be ascertained, the present experiments are the first to show the presence of kininase activity in tissue as distinct from blood. When kininase activity was found in normal guinea-pig lung perfusate, two aspects were considered - (a) investigation of some of the characteristics of the active substance which may

contribute to its future identification; and (b) whether anaphylactic shock altered the kininase activity. Bradykininogen depletion in anaphylaxis suggests that raised bradykinin levels in anaphylaxis are chiefly due to increased formation. The increased bradykinin-forming capacity of post-shock lung perfusate could, however, be due in part to reduced kinin destruction. It was found that anaphylaxis did not alter kininase activity materially and apparently steady and spontaneous leakage of kininase occurs from lung; so reduction of kininase activity seems unlikely to contribute to the increased bradykinin-forming capacity of post-shock guinea-pig lung perfusate.

The time-course of destruction of bradykinin when incubated with perfusate suggests a first order reaction typical of an enzyme. The kininase activity is destroyed by heat and is more thermolabile than BKF. Hexadimethrine bromide does not inhibit kininase activity. Since the presence of disodium ethylenediamine tetra-acetic acid (EDTA) during perfusate + bradykinin incubation does not inhibit bradykinin destruction seriously, Ca^{++} probably is not essential for kininase activity. In this regard, the lung kininase activity as studied in the present experiments appears to be different from the plasma kininase activity studied by Armstrong et al. (1955), who found that the decay of bradykinin activity in glass-exposed human plasma was greatly reduced by the presence of disodium EDTA. It has been shown recently that leucocytes contain two types of kininases, one of which is inhibited by EDTA, whereas the

other is not (Erdös et al., 1962; Schwab, 1962). It would seem likely that there are several types of kininase.

Soya bean trypsin inhibitor does not inhibit kininase while it suppresses BKF markedly. It may be possible to utilise this fact in the estimation of kininase activity in a biological fluid which contains kininase and BKF as well as bradykininogen.

In the course of the present experiments, the optimum pH value for kininase activity was found to be between 7.0 and 7.4. There is a sharp drop in kininase activity in the acid side, i.e. below the 6.6 - 6.8 level. As already mentioned, the pH-optimum of BKF between 6.5 and 6.8 may be significant in other pathological conditions in which it is suspected to play a role - e.g. reactive hyperaemia, burn and shock. Thus at pH 6.75 there will be optimum BKF activity and reduced kininase activity so there will presumably be a net gain of bradykinin content attributable solely to pH. This finding thus supports the idea that bradykinin is a physiological regulator in reactive hyperaemia, and that it plays an active role in any tissue subjected to oxygen lack or an excess of acid products, including metabolites of the Krebs cycle, the Cori cycle and products of cell destruction. The bradykinin system is thus seen to be well fitted as a local regulator of blood and lymph flow in damaged or comparatively anoxic tissue, where it can function as a self-limiting homeostatic process.

Apart from smooth muscle contraction, another possible contribution of bradykinin to bronchial pathology was considered. Increased secretion from the mucous glands of

the bronchial tree plays an important part in the pathological changes in bronchial asthma. Such increase is not satisfactorily explained by histamine or 5-hydroxytryptamine, and the participation of bradykinin was considered. Since there is no experimental method for estimating rather rapid or short-lived changes in experimental bronchial mucosecretion, a method was devised. This was based on the premise that when the bronchial tree is in contact with a physiological solution, any increase in mucosecretion due to the presence of a drug in the solution would be reflected in an increased mucoprotein content in the solution. To ensure proper dispersion of the mucoprotein in the solution, the lung is inflated and deflated rhythmically. However, the powerful bronchoconstriction produced by bradykinin hindered the inflation and deflation and withdrawal of samples. Some of the mucoprotein was probably trapped in those bronchioles cut off from the sampling fluid. Doses of 2.5- 3 ng/ml were employed and this was an unexpected demonstration of the bronchoconstrictor effect of bradykinin in vitro. After the administration of bradykinin there was a slight rise in the mucoprotein content, but not more than in the serially collected control samples. Histological examination likewise did not show any evidence of increased mucosecretion after bradykinin administration in vivo. These experiments were abandoned because trials to improve the method seemed likely to require more time than could be spared. The results are inconclusive, but the problem is of sufficient interest to warrant further study.

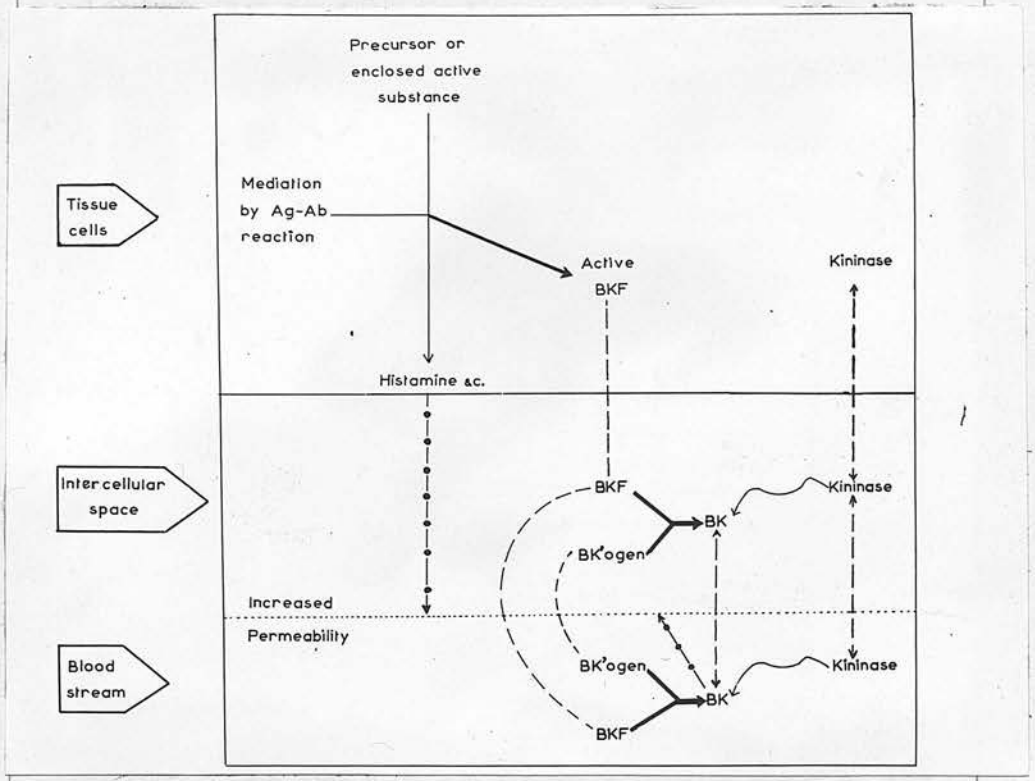


Fig. 30: Postulated scheme of anaphylactic release of bradykinin; BK = bradykinin; BKF = bradykinin-forming activity; BK'ogen = bradykininogen; ⟶ = conversion; – – – = transport; —•—•→ = biological effect; ∿→ = destruction.

From the present experiments one may follow some of the steps which lead to bradykinin formation in anaphylaxis. This is shown schematically in figure 30. One would visualise that antigen-antibody reaction releases BKF from tissue; at the same time, released histamine increases vascular permeability, and brings BKF in contact with bradykininogen in the interstitial spaces, and perhaps within the smaller blood vessels also. Bradykinin formation would follow and further facilitate interaction between BKF and bradykininogen by maintaining increased vascular permeability. The formed bradykinin probably travels freely through capillary walls so that it can act on the plain muscle of both blood vessels and organs. Formation of bradykinin progresses

pari passu with bradykinin destruction by kininase. The balance of the two processes would determine the net amount of bradykinin present. Immediately following the explosive liberation of BKF in the first minutes after challenge, formation exceeds destruction, and bradykinin level in the body rises; later, when BKF activity decreases, the formed bradykinin is rather quickly inactivated by the kininase present in blood and tissue. When BKF release proceeds for a considerable period, as presumably occurs in shock due to retarded challenge, there is a considerable turnover of bradykinin, which is reflected in prolonged bradykinin activity. The depletion of bradykininogen is so great that eventually bradykinin formation must be slowed if the release of BKF persists.

At present, comments on the possible role of bradykinin in clinical allergic states must necessarily be speculative, since there is a marked species variation in anaphylaxis. In bronchial asthma, the comparative therapeutic failure of antihistamine drugs suggests the participation, besides histamine, of other mediators. The non-histaminic component of bronchoconstriction in asthma is probably mostly due to SRS-A (Brocklehurst, 1960), but bradykinin may also contribute to the overall picture since it can cause wheezing and dyspnoea in man (Herxheimer and Streseman, 1961). It is not clear whether bradykinin contributes to increased secretion of bronchial mucous glands in asthma. Bradykinin may also contribute to prolonged allergic conditions in man by virtue of its effect

on the tonus and permeability of blood vessels. It is thus likely to be very largely concerned in oedematous conditions which persist much longer than would be expected if histamine were the responsible agent, and where anti-histaminic drugs are not very active. These conditions include oedema of the bronchial mucosa in asthma, often that of the nasal mucosa in hay fever, as well as chronic urticarial conditions.

Bradykinin is known to produce pain and irritation when applied to the exposed cutaneous sensory nerve endings of a blister base (Armstrong et al., 1957); it is therefore possible that it makes some contribution not only to the pain and irritation of burns, but also to cutaneous allergic states. Furthermore, sensory endings in the lung and other organs may also be stimulated by bradykinin and lead to reflex rearrangement of the autonomic control of the organ involved.

It is clear from the evidence obtained in the course of the present investigation that bradykinin is released during anaphylaxis. Does it have a major role to play, or is it in the nature of a pathological artefact or byproduct? The situation varies from species to species, and in any case an evaluation of the contribution of bradykinin to the overall picture must await the discovery of a suitable antagonist. In the guinea-pig, the quantities released must be expected to influence the cutaneous and vascular features of anaphylaxis, and may

participate in the bronchial effect also. It may well play a more important role in the rat, which cannot be significantly protected from anaphylactic shock by antagonists to 5-hydroxytryptamine and histamine.

The involvement of bradykinin in anaphylaxis may help to explain some of the "anomalous" phenomena in anaphylaxis which cannot be explained by histamine. SRS-A explains some of these effects but bradykinin may well be suited as the responsible agent in several others, particularly those concerning blood vessels.

In guinea-pigs antihistaminic agents offer considerable protection against anaphylaxis, but even in mepyramine-protected animals some distress occurs which may lead to collapse in strongly sensitised animals. Kallos and Pagel (1937) found that eosinophil leucocytes accumulate in large numbers in tissue in which an immediate-type allergic reaction has taken place, though this follows the main reaction. Injection of histamine or heparin can only mimic this feature weakly or not at all. Transplantation of lungs taken from animals dying of anaphylaxis to normal guinea-pigs caused a great increase in eosinophil leucocytes a few hours later. This would suggest the participation of a substance formed in the tissue (Samter, Kofoed and Pieper, 1953). This substance is likely to be bradykinin, since it is known to produce migration of leucocytes.* Inderbitzin (1957) found that in mepyramine-protected guinea-pigs increase in capillary permeability

* This does not necessarily imply that bradykinin is selectively chemotactic for eosinophils.

was not reduced during PCA, suggesting that some substance other than histamine took a major part in the reaction. Further, proteolytic agents were found to produce an increase in permeability, indicating that products of protease activity such as bradykinin are likely to be involved. Isolated guinea-pig uterus from a sensitised animal contracts when antigen is brought in contact, even though the preparation is refractory to histamine following prolonged contact with histamine (Schild, 1936). As SRS-A does not contract this preparation, bradykinin may be the active agent, provided that bradykininogen was available from tissue. This could be investigated by treating the tissue with trypsin or Bothrops venom to remove bradykininogen before challenging the tissue.

Some anaphylactic phenomena in rat cannot be explained with histamine. Sanyal and West (1958) found that pretreatment with mepyramine or BOL or both did not significantly reduce the severity of anaphylactic shock. They concluded that neither histamine nor 5-hydroxytryptamine plays a role in anaphylaxis in rat. Similar observations were made with isolated sensitised rat uterus. This preparation is not contracted by histamine or SRS-A. Kellaway (1930) showed that contact with antigen contracts sensitised rat uterus, which means that a substance other than histamine is involved. Brocklehurst (1958) found that this phenomenon occurred even when 5-hydroxytryptamine activity had been suppressed with LSD, which indicates that the activity is not due to 5-hydroxytryptamine. Bradykinin

may be the substance concerned, if bradykininogen is available from tissue. Furthermore, Brocklehurst, Humphrey and Perry (1955, 1960) found that when the participation of histamine and 5-hydroxytryptamine were excluded by depletion and the presence of antagonists, increased cutaneous vascular permeability in PCA was not abolished. Inderbitzin, Dobric and Goetschmann (1959) also found that the degree of increase in capillary permeability is not correlated with the amount of histamine released. The most likely explanation of these cutaneous reactions is the participation of a substance having the properties of bradykinin.

The present work suggests several lines for further investigation.

(1) A suitable bradykininogen-depleting procedure should be developed, and the effect of this in vivo, and on isolated tissue observed.

(2) Further work on the nature and mode of action of BKF is indicated; it might be useful to examine the effect of analgesic-antipyretics (particularly phenylbutazone and acetylsalicylic acid), corticosteroids, enzyme inhibitors and various competitive substrates.

(3) Additional study of the release of BKF itself, specially with regard to the effect of heat, pH, calcium-dependence, and enzyme inhibitors as also examination of the influence of succinic acid and maleic acid, which enhance anaphylactic release of histamine, and the effect

of general metabolic stimulants like thyroxine.

General experiments extending the work to other species and tissue is desirable, particularly "shock" organs and human tissue if it can be obtained without damage or enzyme activation. The role of bradykinin in clinical allergic states, particularly asthma should be investigated in view of its possible clinical significance. As a beginning, estimation of blood bradykinin level during status asthmaticus and perfusion of sensitised human lung, if obtainable, is suggested.

BIBLIOGRAPHY

Abelous, J.E. and Bardier, E. (1909): C. R. Soc. Biol., Paris, 66, 511.

Alberty, J. and Schiede, M. (1953): Arch. int. Pharmacodyn., 96, 28.

Albrechtsen, O.K. (1959): Acta Physiol. scand., 47, Suppl. 165, 13.

Alfonso, S., Rowe, G.G., Castillo, C.A., Lowe, W.C. and Crumpton, C.W. (1962): Fed. Proc., 21, 94.

Amin, A.H. (1953): The distribution of 5-hydroxytryptamine and substance P in the nervous system. Ph.D. thesis, University of Edinburgh.

Armstrong, D., Dry, R.M., Keele, C.A. and Markham, J.W. (1953): J. Physiol., London, 120, 326.

Armstrong, D., Hobbiger, F., Jepson, J.B. and Keele, C.A. (1953): Abstr. XIX Internat. Physiol. Congr., p. 173.

Armstrong, D., Jepson, J.B., Keele, C.A. and Stewart, J.W. (1955): J. Physiol., London, 129, 80P.

Armstrong, D., Keele, C.A., Jepson, J.B. and Stewart, J.W. (1954): Nature, London, 174, 791.

Armstrong, D., Jepson, J.B., Keele, C.A. and Stewart, J.W. (1957): J. Physiol., London, 135, 350.

Armstrong, D. and Stewart, J.W. (1960): J. Physiol., London, 154, 19P.

Armstrong, D. and Stewart, J.W. (1962): Brit. J. Pharmacol., 18, 6.

Aronson, H. (1912): Berl. klin. Wschr., 49, 642.

Astrup, T. and Sterndorff, I. (1952): Proc. Soc. exp. Biol., N.Y., 81, 675.

Austen, K.F. and Brocklehurst, W.E. (1961a): J. exp. Med., 113, 521.

Austen, K.F. and Brocklehurst, W.E. (1961b): J. exp. Med., 113, 541.

Austen, K.F. and Brocklehurst, W.E. (1961c): J. exp. Med., 114, 29.

Beloff, A. and Peters, R.A. (1945): J. Physiol., London, 103, 461.

Benditt, E.P. and Arase, M. (1959): J. exp. Med., 110, 451.

Benditt, E.P., Wong, A.L., Arase, M. and Roeper, E. (1955): Proc. Soc. exp. Biol., N.Y., 90, 303.

Beraldo, W.T. (1950): Amer. J. Physiol., 163, 283.

Beraldo, W.T. (1952): Amer. J. Physiol., 171, 371.

Beraldo, W.T., Feldberg, W. and Hilton, S.M. (1956): J. Physiol., London, 133, 558.

Berde, B. and Cerletti, A. (1961): Helv. physiol. acta., 19, C7.

Berde, B. and Saameli, K. (1961): Nature, London, 191, 83.

Bhoola, K.D., Calle, J.D. and Schachter, M. (1960): J. Physiol., London, 152, 75.

Bhoola, K.D., May May Yi, Rose, Morely, J. and Schachter, M. (1961): J. Physiol., London, 159, 34P.

Biedl, A. and Kraus, R. (1909): Wien. klin. Wschr., 22, 363.

Biedl, A. and Kraus, R. (1911): Dtsch. med. Wschr., 37, 1300.

Boissonas, R.A., Guttmann, St. and Jaquenoud, P.-A. (1960a): Helv. chim. acta, 43, 1349.

Boissonas, R.A., Guttmann, St. and Jaquenoud, P.-A. (1960b): Helv. chim. acta, 43, 1481.

Boissonas, R.A., Guttmann, St., Jaquenoud, P.-A., Konzett, H. and Sturmer, E. (1960): Experientia, 16, 326.

Boréus, L.O. and Chakravarty, N. (1960): Acta physiol. scand., 48, 315.

Braun, C., Guzman, F., Horton, E.W., Lim, R.K.S. and Potter, G. (1960): J. Physiol., London, 155, 13P.

Braun-Falco, O. and Salfeld, K. (1959): Nature, London, 183, 51.

Brocklehurst, W.E. (1953): J. Physiol., London, 120, 16P.

Brocklehurst, W.E. (1958): The action of 5-hydroxytryptamine on smooth muscle. In: 5-hydroxytryptamine. ed. G.P. Lewis, London. Pergammon Press.

Brocklehurst, W.E. (1958a): The formation and release of a slow-reacting substance in the course of the anaphylactic reaction. Ph.D. Thesis, London.

Brocklehurst, W.E. (1960): J. Physiol., London, 151, 416.

Brocklehurst, W.E. (1962): Slow reacting substance and related compounds. In: Progress in allergy, vol. VI. ed. P. Kallós. Karger, Basel.

Brocklehurst, W.E., Humphrey, J.H. and Perry, W.L.M. (1960): J. Physiol., London, 150, 489.

Bronfenbrenner, J. (1915): J. exp. Med., 21, 480.

Burdon, K.L. (1952): Mechanisms of hypersensitivity and their significance in relation to certain aspects of the radiation syndrome: a critical review. United States Air Force, Project number 21-3501-0004, report number 1.

Campbell, D.H. and Nicoll, P.A. (1940): J. Immunol., 39, 102.

Čapek, R. (1961): Biochem. Pharmacol., 8, 178.

Chakravarty, N. (1960): Acta physiol. Scand., 48, 167.

Chakravarty, N. and Uvnäs, B. (1960): Acta physiol. scand., 48, 302.

Chapman, L.F., Goodell, H. and Wolff, H.G. (1959a): Arch. Neurol., 1, 557.

Chapman, L.F., Goodell, H. and Wolff, H.G. (1959b): Fed. Proc., 18, 25.

Chapman, L.F., Ramos, A., Corrado, A., Fortes, V. and Symmes, D. (1960): Arch. Neurol., 3, 43.

Chapman, L.F. and Wolff, H.G. (1958): Science, 128, 1208.

Chapman, L.F. and Wolff, H.G. (1959): Arch. intern. Med., 103, 86.

Cliffton, E.E. (1952): J. Lab. clin. Med., 39, 105.

Collier, H.O.J., Holgate, J.A., Schachter, M. and Shorley, P.G. (1959): J. Physiol., London, 149, 54P.

Collier, H.O.J., Holgate, J.A., Schachter, M. and Shorley, P.G. (1960): Brit. J. Pharmacol., 15, 290.

Collier, H.O.J. and Shorley, P.G. (1960): Brit. J. Pharmacol., 15, 601.

Coppen, A.J. and Lewis, G.P. (1961): J. Neurochem., 7, 155.

Dale, H.H. (1913): J. Pharmacol., 4, 167.

Dale, H.H. and Laidlaw, P.P. (1910): J. Physiol., London, 41, 318.

Dale, H.H. and Laidlaw, P.P. (1912): J. Pharmacol., 4, 75.

Damgaard, E. and Ungar, G. (1952): Amer. J. Physiol., 171, 717.

Diniz, C.R., Carvalho, I.F., Ryan, J. and Rocha e Silva, M. (1961): Nature, London, 192, 1194.

Doepffner, W. and Cerletti, A. (1958): Int. Arch. Allergy, Basel, 12, 89.

Dresser, D.W. (1960): Immunology, 3, 289.

Eagle, H., Johnston, C.G. and Ravdin, I.S. (1937): Bull. Johns Hopk. Hosp., 60, 428.

Edery, H. and Lewis, G.P. (1962a): J. Physiol., London, 160, 20P.

Edery, H. and Lewis, G.P. (1962b): J. Physiol., London, in press.

Edman, K.A.P. and Mongar, J.L. (1961): J. Physiol., London, 157, 40P.

Eisen, V. (1961): Brit. J. Pharmacol., 18, 6.

Eisen, V. (1961): Modes of formation of human plasma kinin. Ph.D. thesis, London.

Elliott, D.F., Horton, E.W. and Lewis, G.P. (1960): J. Physiol., London, 153, 473.

Elliott, D.F., Lewis, G.P. and Horton, E.W. (1960): Biochem. Biophys. Research Communications, 3, 87.

Erdös, E.G., Renfrew, A.G., Sloane, E.M. and Wohler, J.R. (1962): Enzymatic studies on bradykinin and similar peptides. Abstract, Symp. N.Y. Acad. Sci., held in March, 1962.

Feldberg, W. (1961): Arch. klin. exper. Dermat., 213, 343.

Feldberg, W. and Guimarāis, J.A. (1935): J. Physiol., London, 85, 15.

Feldberg, W. and Paton, W.D.M. (1951): J. Physiol., London, 114, 490.

Fink, M.A. (1956): Proc. Soc. exp. Biol., N.Y., 92, 673.

Fink, M.A. and Gardiner, C.E. (1958): Proc. Soc. exp. Biol., N.Y., 97, 554.

Fox, C.L., Einbinder, J.M. and Nelson, C.T. (1958): Amer. J. Physiol., 192, 241.

Fox, R.H., Goldsmith, R., Kidd, D.J. and Lewis, G.P. (1960): J. Physiol., London, 157, 589.

Fox, R.H. and Hilton, S.M. (1958): J. Physiol., London, 142, 219.

Freund, H. (1920): Arch. exp. Path. Pharmak., 86, 266; 88, 39.

Freund, H. (1921): Arch. exp. Path. Pharmak., 91, 272.

Frey, E.K. and Kraut, H. (1928): Arch. exp. Path. Pharmak., 133, 1.

Frey, E.K., Kraut, H. and Werle, E. (1950): Kallikrein Padutin. Stuttgart, Enke.

Friedberger, E. (1909): Z. ImmunForsch., 2, 208.

Friedberger, E. (1910): Z. ImmunForsch, 4, 636.

Friedemann, U. (1909): Z. ImmunForsch., 2, 591.

Gaddum, J.H. (1936): Gefässerweiternde Stoffe der Gewebe. Leipzig, Georg Thieme Verlag.

Gaddum, J.H. (1955): Discussion: In polypeptides which stimulate plain muscle. Edinburgh, E. & S. Livingstone.

Geiger, W.B. (1952): J. Immunol., 69, 597.

Glenner, G.G. and Cohen, L.A. (1960): Nature, London, 185, 846.

Goldhaber, P., Cornman, I. and Ormsbee, R.A. (1947): Proc. Soc. exp. Biol., N.Y., 66, 590.

Green, N.M. (1953): J. biol. Chem., 205, 535.

Guth, P.S. (1959): Brit. J. Pharmacol., 14, 549.

Guth, P.S. (1960): Discussion: In Polypeptides which affect smooth muscle and blood vessels. Ed. M. Schachter. London, Pergamon Press.

Guzman, F., Braun, C. and Lim, R.K.S. (1962): Arch. int. Pharmacodyn., 136, 353.

Hamberg, U. and Rocha e Silva, M. (1954): Acta physiol., scand., 30, 215.

Hawkins, O.F. and Rosa, L. (1959): Int. Arch. Allergy, Basel, 14, 312.

Hayashi, H. (1956): Mie med. J., 6, 195.

Henriques, O.B., Picarelli, Z.P. and Oliveira, M.C.F. (1962): Biochem. Pharmacol., 11, 707.

Herberts, G. (1949): Acta med. Soc. Uppsal., 54, 387.

Herberts, G. (1955): Acta med. Soc. Uppsal., 60, 246.

Herberts, G. (1955): Acta allergol., 9, 167.

Herberts, G. (1958): Acta allergol., 12, 58.

Herxheimer, H. (1955): J. Physiol., London, 128, 435.

Herxheimer, H. and Streseman, E. (1961): J. Physiol., London, 158, 38P.

Hilton, S.M. and Lewis, G.P. (1956): J. Physiol., London, 134, 471.

Hilton, S.M. and Lewis, G.P. (1958): J. Physiol., London, 144, 532.

Högberg, B. and Uvnäs, B. (1960): Acta physiol. scand., 48, 133.

Holdstock, D.J., Mathias, A.P. and Schachter, M. (1957): Brit. J. Pharmacol., 12, 149.

Horton, E.W. (1958): Polypeptides which stimulate plain muscle. Ph.D. thesis, Edinburgh.

Horton, E.W. (1959): J. Physiol., London, 148, 267.

Horton, E.W. and Lewis, G.P. (1960): J. Physiol., London, 149, 477.

Humphrey, J.H. and Jaques, R. (1954): J. Physiol., London, 124, 305.

Humphrey, J.H. and Jaques, R. (1955): J. Physiol., London, 128, 9.

Inderbitzin, T. (1955): Int. Arch. Allergy, Basel, 7, 140.

Inderbitzin, T. (1961): Int. Arch. Allergy, Basel, 18, 85.

Inderbitzin, T. and Craps, L. (1957): Dermatologica, Basel, 114, 208.

Inderbitzin, T., Dobric, V. and Goetschmann, I. (1959): Int. Arch. Allergy, Basel, 15, 270.

Inderbitzin, T. and Goetschmann, I. (1959): Int. Arch. Allergy, Basel, 17, 323.

Jaques, R. and Waters, E.T. (1941): J. Physiol., London, 99, 454.

Jobling, J.W. and Petersen, W. (1914): J. exp. Med., 20, 37.

Jorpes, J.E., Holmgren, H. and Wilander, O. (1937): Z. mikr.-anat. Forsch., 42, 279.

Kabat, E.A. and Benacerraf, B. (1949): J. Immunol., 62, 97.

Kallos, P. and Pagel, W. (1937): Acta med. scand., 91, 292.

Katz, G. (1940): Science, 91, 221.

Katz, G. and Cohen, S. (1941): J. Amer. med. Ass., 117, 1782.

Kellaway, C.H. (1930): Brit. J. exp. Path., 11, 72.

Kellaway, C.H. and Trethewie, E.R. (1940): Quart. J. exp. Physiol., 30, 121.

Kidd, D.J. and Lewis, G.P. (1960): J. Physiol., London, 154, 16P.

Konzett, H. and Sturmer, E. (1960): Brit. J. Pharmacol., 15, 544.

Kraut, H. and Korbel-Enkhardt (1957): Hoppe-Seyl. Z., 309, 243.

Kuhns, W.J. and Pappenheimer, A.M. (1952): J. exp. Med., 95, 375.

Lecomte, J. (1960): C. R. Soc. Biol., Paris, 154, 2389.

Lecomte, J. and Troquet, J. (1960): C. R. Soc. Biol., Paris, 154, 1115.

Lewis, G.P. (1959): J. Physiol., London, 147, 458.

Lewis, G.P. (1960): Physiol. Rev., 40, 647.

Lewis, J.H. and Ferguson, J.H. (1950): J. clin. Invest., 29, 486.

Lewis, G.P. and Work, T. (1957): J. Physiol., London, 135, 7P.

Lim, R.K.S.(1960): Ann. N. Y. Acad. Sci., 86, 73.

Long, G. and Penny, I.F. (1957): Biochem. J., 65, 382.

Lowell, F.C., Franklin, W., Schiller, I.W. and Follensby, E.M. (1956): J. Allergy, 27, 369.

Lutwak-Mann, C. (1942): Biochem. J., 36, 706.

MacFarlane, R.G. and Pilling, J. (1946): Lancet, ii, 562.

MacIntosh, F.C. and Paton, W.D.M. (1949): J. Physiol., London, 109, 190.

McIntire, F.C. (1956): The mode of histamine binding in animal tissues. In: Ciba Foundation symposium on histamine. London, Churchill.

McIntire, F.C., Roth, L.W. and Sproull, M. (1950): Proc. Soc. exper. Biol., N.Y., 73, 605.

Mackay, M.E., Miles, A.A., Schachter, M. and Wilhelm, D.L. (1953): Nature, London, 172, 714.

Margolis, J. (1958): J. Physiol., London, 144, 1.

Miles, A.A. and Wilhelm, D.L. (1955): Brit. J. exp. Path., 36, 71.

Mongar, J.L. and Schild, H.O. (1957a): J. Physiol., London, 135, 301.

Mongar, J.L. and Schild, H.O. (1957b): J. Physiol., London, 135, 320.

Mongar, J.L. and Schild, H.O. (1958): J. Physiol., London, 140, 272.

Mongar, J.L. and Schild, H.O. (1962): Physiol. Rev., 42, 226.

Monkhouse, F.C., Fidlar, E. and Barlow, J.C.D. (1952): Amer. J. Physiol., 169, 712.

Mota, I. (1957): Brit. J. Pharmacol., 12, 453.

Mota, I. and Ishii, T. (1960): Brit. J. Pharmacol., 15, 82.

Müllertz, S. (1956): Acta physiol. scand., 38, Suppl. 130.

Müllertz, S. and Lassen, M. (1953): Proc. Soc. exp. Biol., N.Y., 82, 264.

Neville-Jones, D. and Peters, R.A. (1948): Biochem. J., 43, 303.

Nicolaides, E.D. and De Wald, H.A. (1962): Synthesis of some kinin analogues. Abstract, Symp. N.Y. Acad. Sci., held in March 1962.

Northover, B.J. and Subramanian, G. (1961): Brit. J. Pharmacol., 17, 107.

Parratt, J.R. and West, G.B. (1956): J. Physiol., London, 132, 40P.

Parratt, J.R. and West, G.B. (1957a): J. Physiol., London, 137, 169.

Parratt, J.R. and West, G.B. (1957b): J. Physiol., London, 137, 179.

Parratt, J.R. and West, G.B. (1957c): J. Physiol., London, 139, 27.

Parratt, J.R. and West, G.B. (1958): Brit. J. Pharmacol., 13, 65.

Picarelli, Z.P., Henriques, O.B. and Oliveira, M.C. (1962): Experientia, 18, 77.

Pierce, J.V. and Webster, M.E. (1961): Biochem. biophys. Research Communications, 5, 353.

Portier, P. and Richet, C. (1902): C. R. Soc. Biol., Paris, 54, 170.

Pribram, H. and Herrnheiser, S. (1920): Biochem. Z., 111, 30.

Rapp, H.J. (1961): J. Physiol., London, 158, 35P.

Rocha e Silva, M. (1939): C. R. Soc. Biol., Paris, 130, 186.

Rocha e Silva, M. (1940): Arch. exp. Path. Pharm., 194, 335.

Rocha e Silva, M. (1943): J. Pharmacol., 77, 198.

Rocha e Silva, M. (1960): Biochemistry and Pharmacology of bradykinin. In: Polypeptides which affect smooth muscles and blood vessels. ed. M. Schachter. London, Pergamon Press.

Rocha e Silva, M. and Antonio, A. (1960): Med. exp., 3, 371.

Rocha e Silva, M., Beraldo, W.T. and Rosenfeld, G. (1949): Amer. J. Physiol., 156, 261.

Rocha e Silva, M., Corrado, A.P. and Ramos, A.O. (1960): J. Pharmacol., 128, 217.

Rocha e Silva, M. and Holzhacker, E.L. (1959): Arch. int. Pharmacodyn., 122, 168.

Rocha e Silva, M. and Rosenthal, S.R. (1961): J. Pharmacol., 132, 110.

Rocha e Silva, M., Scroggie, A.E., Fidlar, E. and Jaques, L.B. (1949): Proc. Soc. exp. Biol., N.Y., 64, 141.

Rowley, D.A. and Benditt, E.P. (1956): J. exp. Med., 103, 399.

Saameli, K. and Eskes, T. (1962): Fed. Proc., 21, 400.

Samter, M., Kofoed, M.A. and Pieper, W. (1953): Blood, 8, 1078.

Sanyal, R.K. and West, G.B. (1958): J. Physiol., London, 144, 525.

Schachter, M. (1955): J. Physiol., London, 129, 30P.

Schachter, M. (1956): Brit. J. Pharmacol., 11, 111.

Schachter, M. (1960): Some properties of kallidin, bradykinin and wasp venom. In: Polypeptides which affect smooth muscles. ed. M. Schachter. London, Pergamon Press.

Schayer, R. (1956): Discussion. In: Ciba Foundation Symposium on histamine. London, Churchill.

Schild, H.O. (1936): J. Physiol., London, 86, 51P.

Schild, H.O., Hawkins, D.F., Mongar, J.L. and Herxheimer, H. (1951): Lancet, ii, 376.

Schultz, W.H. (1910): J. Pharmacol., 1, 546.

Schwab, J. (1962): Nature, London, 195, 345.

Scroggie, A.E. and Jaques, L.B. (1949): J. Immunol., 62, 103.

Secker, J. (1934): J. Physiol., London, 81, 81.

Smith, W.G. (1962): Life Sciences, in press.

Sturmer, E. and Cerletti, A. (1961): Helv. physiol. acta, 19, C32.

Tagnon, H.J. and Peterman, M.L. (1949): J. clin. Invest., 28, 814.

Tella, A. and Maegraith, B.G. (1962): Trans. roy. Soc. trop. Med. Hyg., 56, 6.

Ungar, G. and Damgaard, E. (1955): J. exp. Med. 101, 1

Ungar, G. and Mist, S.H. (1949): J. exp. Med., 90, 39.

Ungar, G. and Parrot, J.L. (1936): C. R. Soc. Biol., Paris, 123, 676.

"

Uvnas, B. and Thon, I.-L. (1961): Experimental Cell Research, 23, 45.

van Arman, C.G. (1955): Interrelationships among some peptide precursors. In: Polypeptides which stimulate plain muscle. ed. J.H. Gaddum. Edinburgh, Livingstone.

van Arman, C.G. and Miller, L.M. (1961): J. Pharmacol., 131, 366.

Van Den Driessche, R. (1960): Discussion. In: Polypeptides which affect smooth muscle and blood vessels. ex. M. Schachter. London, Pergamon Press.

van Winkle, W., Jr. (1941): Proc. Soc. exp. Biol., N.Y., 46, 220.

Vaughan, V.C. and Wheeler, S. (1907): J. infect. Dis., 4, 476.

Waaler, B.A. (1961): J. Physiol., London, 157, 475.

Waalkes, T.P. and Coburn, H. (1959): J. Allergy, 30, 394.

Webster, M.E. and Pierce, J.V. (1960): J. Pharmacol., 130, 484.

Webster, M.E. and Pierce, J.V. (1961): Proc. Soc. exp. Biol., N.Y., 107, 186.

Weddell, G. and Pallie, W. (1954): Peripheral circulation in man. Ciba Foundation symposium. London, Churchill.

Weiser, R.S. (1957): J. Allergy, 28, 475.

Weissbach, H.T., Waalkes, T.P. and Udenfriend, S. (1957): Science, 125, 235.

Werle, E. (1934): Biochem. Z., 273, 291.

Werle, E. (1955): Chemistry and pharmacology of kallikrein and kallidin. In: Polypeptides which stimulate plain muscle. ed. J.H. Gaddum. Edinburgh, Livingstone.

Werle, E. (1960): Kallikrein, kallidin and related substances. In: Polypeptides which affect smooth muscles and blood vessels. ed. M. Schachter. London, Pergamon Press.

Werle, E., Appel, W. and Happ, E. (1958): Arch. exp. Path. Pharmak., 234, 364.

Werle, E., Götze, W. and Keppler, A. (1937): Biochem. Z., 289, 217.

Werle, E. and Maier, L. (1952): Biochem. Z., 322, 414.

Werle, E., Maier, L. and Löffler, F. (1951): Biochem. Z., 321, 172.

Werle, E. and Vogel, R. (1958): In: Leistungen und Ergebnisse der neuzeitlichen Chirurgie, Stuttgart.

Werle, E. and Vogel, R. (1960): Arch. int. Pharmacodyn., 126, 171.

Werle, E. and Vogel, R. (1960a): Unpublished findings, referred in Werle (1960).

Winzler, R.J. (1955): Determination of serum glycoprotein. In: Methods of Biochemical Analysis, vol. 2. ed. D. Glick. New York, Interscience Publishers.

Yoffey, J.M. and Courtice, F.C. (1956): Lymphatics, lymph and lymphoid tissue (2nd edition). London, Arnold.

ACKNOWLEDGMENTS

I am grateful to Prof. W.L.M. Perry, O.B.E., M.D., D.Sc., for his sustained interest and encouragement, and to Dr. W.E. Brocklehurst, Ph.D., for his interest and advice at every stage of the investigation. Much help was obtained from Miss Alice Urquhart and Mr. Ian Dow in the preparation of the illustrations.

The award of a Colombo Plan Fellowhip by the United Kingdom Government for the duration of the investigation is gratefully acknowledged.

GIFTS OF MATERIAL

I am grateful to the following people for gifts of material: Dr H.O.J. Collier, Parke Davis Research Laboratories, Hounslow, Middlesex for synthetic bradykinin; Mr David Colquhoun, Department of Pharmacology, University of Edinburgh Medical School, for anti-BSA rabbit antibody; Dr H. Holgate, Sandoz Products Ltd., Wigmore Street, London, W.1. for BOL 148; Professor H.O. Schild, University College, London for a sample of the bradykinin used as a standard by Professor M. Rocha e Silva; and Dr R. Wien, May and Baker Ltd., Dagenham, Essex, for mepyramine maleate.